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14. ABSTRACT

Numerous efforts have been made to identify proteins that are either overexpressed or expressed in an aberrant form favoring selective growth advantage or metastasis of breast cancer cells.

NF-κB/Rel is a family of regulators of gene expression that has been found to play important roles for cell proliferation and survival. Our laboratory observed that aberrant increased activity of NF-κB/Rel occurs in breast cancer cells, but not in the normal counterpart breast epithelial cells. Inhibition of NF-κB/Rel activity in breast cancer cells cultivated *in vitro* led to their death. Results observed by other laboratories similarly showed that many hematopoietic and solid tumors display increased NF-κB/Rel activity, supporting our findings.

We have begun to characterize the mechanisms of NF-κB/Rel increased activity in breast cancer cells. We are currently assessing whether proteins of the cell's machinery that are known to regulate NF-κB/Rel activity can be overexpressed in breast cancer cells, and thus leading to increased NF-κB/Rel activity. The results of these studies will provide important information of the potential role and mechanisms of NF-κB/Rel factor overexpression in the etiology of breast cancers. Importantly, identification of these proteins may identify new targets for treatment of breast cancer or biomarkers for analysis of disease progression.

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Roles of IκB-α Protein Kinases in Activation of NF-κB in Breast Cancer

TECHNICAL ABSTRACT

Aberrant activation of NF-κB transcription factors has been implicated in the pathogenesis of breast cancer. To elucidate the mechanism of NF-κB activation, we focused our attention on the kinases that affect IκB stability. We previously demonstrated elevated activity of IKK1, IKK2 and protein kinase CK2 (formerly casein kinase 2) in primary human breast cancer specimens and cell lines in culture. A novel inducible IKK protein termed IKKi or IKKε has only recently been characterized as a potential NF-κB activator. Here we provide evidence that implicates IKKi in the pathogenesis of breast cancer. We report that primary human tumor specimens express IKKi. Hs578T breast cancer cells and D3-1 carcinogen transformed breast epithelial cells showed higher IKKi expression and activity when compared to untransformed MCF-10F breast epithelial cells. Interestingly, IKKi expression correlated with CK2α activity in mammary glands and breast tumors of MMTV-CK2α and MMTV-CK2α/MMTV-c-*rel* vs MMTV-c-*rel* transgenic mice. Protein kinase CK2 is a hetero-tetramer consisting of two α or α' subunits and two β subunits. CK2α overexpression and CK2α stable expression in untransformed cells led to increases in IKKi at both the mRNA and protein level. Conversely, reduction of CK2α activity with the pharmacological inhibitor apigenin or upon transfection of a CK2 kinase inactive subunit reduced IKKi mRNA and protein expression in Hs578T breast cancer cells, which display high basal levels of CK2 activity. In *in vitro* kinase assays, IKKi phosphorylated both GST-IκB-α and GST-IκB-β substrates; although, ectopic overexpression of IKKi in untransformed cells led to a reduction in levels of IκB-α, but not of IκB-β or IκB-ε, and to increased NF-κB reporter activity. Lastly, expression of kinase inactive IKKi mutants reduced NF-κB reporter activity in Hs578T and NF639 Her-2/neu-driven breast cancer cells, and increased steady-state levels of IκB-α, but not IκB-β. Taken together these result indicate that IκB-α is the preferred *in vivo* target of IKKi. Overall, these studies show a functional role for IKKi, which is induced by the protein kinase CK2, as a mediator of NF-κB activation in pathogenesis of breast cancer, and suggest IKKi may be a novel target in the treatment of breast cancer.

RESEARCH AIMS

Aim 1. Determine the levels of IKKα, IKKβ and CK2 kinases in breast cancer cells, Month 1-2

a. Evaluate mRNA expression levels.
b. Evaluate protein expression levels.

Aim 2. Evaluate activities of the IKKα, IKKβ and CK2 kinases in breast cancer cells, Month 1-12

a. Perform kinase assays using mutant and wild-type IκB-α fusion protein substrates.
b. Evaluate IKKα, IKKβ, and CK2 kinase activity in human and mouse breast cancer cells, and tissues samples from multiple breast cancer patients.

Aim 3. Elucidate the functional roles of IKKα, IKKβ, and CK2 kinases in breast cancer cell proliferation and survival, Month 1-12

a. Make retroviral vectors necessary for sense and anti-sense expression of the CK2α and β subunits, sense expression of wild-type and dominant negative IKKα and β, and wild-type or mutated IκB-α proteins.
b. Introduce retroviral and/or expression vectors in mammary epithelial cells or mammary tumor cells and select stable cell lines.
c. Investigate the effects of inhibition or overexpression of IKKα, IKKβ, and CK2 kinases in NF-κB binding and transcriptional activity, IκB-α phosphorylation, cell growth survival and transformed phenotype.

Aim 4. Determine the levels and evaluate the activity of the newly discovered IKKε in breast cancer, Month 12-30

a. Characterize IKKi expression in extracts of primary human breast cancer tissue specimens.
b. Characterize IKKi expression in human and mouse breast cancer cell lines.
c. Determine the effects of IKKi on IκB stability and NF-κB activity

Aim 5. Our findings indicated that tumor cells derived from MMTV-CK2 animals display elevated levels of IKKi. Thus, here we will determine the effects of CK2 on IKKi levels and activity in breast cancer**,** Month 18-36

a. Test the effects of inhibition of CK2 activity via apigenin in Hs578T cells on the level of IKKi protein and mRNA expression, and kinase activity.
b. Overexpress CK2 and determine the effects on IKKi levels.
c. Inhibit CK2 using kinase inactive subunits and determine the effects on IKKi expression in breast cancer cells.

BODY

Aims 1-2 were published previously by Dr. Romieu-Mourez (Romieu-Mourez et al., 2001) and in collaboration with Dr. Seldin's lab (Landesman-Bollag et al., 2001) and the PDF of the relevant manuscripts are included as an attachment. Aim 3 was largely characterized by Dr. Romieu-Mourez and published (Romieu-Mourez et al., 2002) and is also included as an attachment. The research Aims 4-5 were undertaken by myself begining in August, 2003 and are accepted for publication in Cancer Research under a manuscript entitled 'IKK-i/IKKε Expression is Induced by CK2 and Promotes Aberrant NF-κB Activation in Breast Cancer Cells'. These aims comprise the majority of this report, with some studies expanded in the manuscript. The key research accomplishments and conclusions encompass all of Aims 1-5. In addition, Dr. Romieu published an additional manuscript (Romieu-Mourez et al., 2003) under DAMD 17-01-1-0158 characeterizing the functional role of c-Rel (which was not part of the original research aims) in breast cancer. This manuscript is also included as an attachment.

Introduction

Nuclear Factor (NF)-κB/Rel is a family of dimeric transcription factors distinguished by the presence of a 300 amino acid region, termed the Rel homology region, which determines much of its function [1]. Classical NF-κB is a heterodimer composed of a RelA (also termed p65) and p50 subunit. In most cells, other than B lymphocytes, NF-κB/Rel proteins are sequestered in the cytoplasm bound to the specific IκB inhibitory proteins, of which IκB-α is the paradigm. While the v-*rel* gene, carried by the highly oncogenic avian reticuloendotheliosis virus strain T (Rev-T) is able to cause tumors in birds, the role of NF-κB in mammalian cancers was less clear for many years [2]. Several oncogenic mammalian viruses were shown to activate NF-κB. For example, the product of the *tax* gene of the HTLV-1 virus activates NF-κB [3], which we showed mediates transactivation of the c-*myc* promoter [4]. Recently, we and others have demonstrated a role for NF-κB/Rel factors in breast cancer [5, 6]. High levels of nuclear NF-κB/Rel were found in human breast tumor cell lines, carcinogen-transformed mammary epithelial cells, and the majority of primary human or rodent breast tumor tissue samples. In contrast, untransformed breast epithelial cells and normal rat mammary glands contained low basal levels [5, 6].

The increased NF-κB/Rel activity in tumor cells has been correlated with a decrease in stability of IκB proteins, in particular of IκB-α, which permits the released NF-κB subunits to translocate into the nucleus [7]. To begin to elucidate the mechanism of this increased turnover, we recently characterized the activity of several kinases implicated in IκB-α turnover. Breast cancer specimens and tumor cells displayed higher levels of activity of either the IκB kinase IKKα or IKKβ proteins (also known as IKK1 and IKK2) and/or the serine/threonine protein kinase CK2 (previously known as casein kinase 2) [8, 9]. Phosphorylation of IκB-α at two serine residues (Ser32 and Ser36) by IKKα or IKKβ, which are present in a large multimeric protein structure termed the IKK complex, which also contains multiple copies of a regulatory subunit NEMO or IKKγ [10, 11], leads to IκB-α ubiquination and subsequent degradation. In the canonical p50/RelA NF-κB induction pathway, IκB-α phosphorylation is mediated upon activation

of the IKK kinase complex. In addition to the N-terminal phosphorylation of IκB-α, it has been shown that CK2-mediated phosphorylation of serine and/or threonine residues [Ser-283, Ser-289, T-291 and Ser-293] within the C-terminal PEST domain of IκB-α also affects its stability [12-15]. CK2 is ubiquitously expressed and thought to be constitutively active as a heterotetrameric protein containing two catalytic (α/α, α'/α or α'/α') and two regulatory (β/β) subunits [16, 17], although recent evidence suggests that CK2 activity can be altered by cellular stress including UV [18, 19]. CK2-mediated phosphorylation of the PEST domain of IκB-α has been implicated in the basal and signal-dependent turnover of IκB-α [12-14, 20]. The mechanisms of the basal degradation of IκB-α are not fully understood, although it has been suggested that it involves IκB complex phosphorylation, ubiquitination and degradation by the 26S proteasome [20] or a calpain-mediated mechanism [21]. These findings have implicated CK2 in control of intrinsic IκB-α stability and thereby in constitutive activation of NF-κB. Importantly, we showed that CK2 levels are elevated in primary human breast cancer specimens as well as in established cell lines [8, 9, 22, 23].

Interestingly, an alternative complex has been identified which uses an inducibile IKK protein initially termed IKKi, which is also known as IKKε (henceforth called IKKi) [24, 25]. IKKi is part of an independent complex containing IKKi, TANK and TRAF [26]. In addition, Tank Binding Kinase, TBK-1, which is highly homologous to IKKi binds to TANK and TRAF and may form an alternative IKK complex consisting of IKKi and TBK-1 [27]. IKKi appears to specifically phosphorylate IκB-α at Ser36 [24, 25]. While the significance of only one phosphorylation event at the N-terminus of IκB-α is not yet entirely clear, it has been hypothesized that it may pre-dispose IκB-α towards Ser32 phosphorylation and subsequent degradation [25]. Furthermore, this alternative IKK complex may be involved in phosphorylation of transcription factors such as c-Jun [26] or NF-κB/Rel directly [28], suggesting that it may be important in multiple signaling pathways. Here, we have explored the role of IKKi in breast cancer and demonstrate activation of IKKi in human breast tumor specimens and in breast cancer cell lines in culture promotes the induction of NF-κB. Interestingly, IKKi expression correlated with CK2α activity in mammary glands and breast tumors of MMTV-CK2α and MMTV-CK2α/c-*rel* vs MMTV-c-*rel* transgenic mice, leading us to hypothesize a role for CK2 in IKKi expression, which was confirmed. This is to our knowledge the first time that elevated IKKi activity has been linked with breast cancer and the novel role of CK2 in this activation elucidated.

METHODS

Human breast cancer specimen analysis. Primary human breast cancer tissue specimens were obtained from patients undergoing surgery for treatment of breast cancer with approval of the Institutional Review Board of Boston Medical Center. Tumors were processed for steroid receptor analysis and any remaining tissue was considered discarded material and used for subsequent analysis of IKKi kinase. Tissues were stored frozen at -75^0C until samples were processed for the nuclear and cytoplasmic protein fractions, as we have described recently [29].

Transgenic mice. Creation of the transgenic MMTV-c-*rel* and MMTV-CK2 transgenic was previously described [22, 29]. Breeding of MMTV-CK2 mice and

MMTV-c-*rel* mice created bi-transgenic MMTV-CK2 X c-*rel* mice. Bi-transgenic mice were screened for transgene expression and monitored for tumor formation (S. Guo, unpublished data). Mice were housed in a 2-way barrier at the Boston University School of Medicine Transgenic mouse facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care. Wild type mice used in the study were all age- and pregnancy-matched. All tissues were frozen immediately after extraction in liquid nitrogen and stored at –80°C.

Cell culture and treatment conditions. The Hs578T tumor cell line, which was derived from a carcinosarcoma, is epithelial in origin, and was grown, as described previously[5]. Where indicated, cells were incubated with 25 or 50 μM apigenin (Sigma) dissolved in DMSO or a similar dilution of DMSO, as control. MCF-10F is a human mammary epithelial cell line established from a patient with fibrocystic disease, which does not display malignant characteristics [30]. D3-1 cells, which were derived by transforming MCF10F cells with 7,12-dimethylbenz(*a*)anthracene (DMBA), were cultured as described previously [30]. Cells were harvested when cultures had reached 75-90% confluence. The MMTV-Her2/neu NF639 cell line was derived from mammary tumors expressing Her2/neu and cultured as previously described [31]. Human HEK293T endothelial derived kidney cells were cultured in DMEM supplemented with 10% FBS (Invitrogen). NIH 3T3 fibroblast cell lines were cultured in DMEM supplemented with 10% FBS (Invitrogen), non-essential amino acids (Invitrogen), glucose-glutamine (Sigma) and penicillin-streptomycin (Invitrogen). NIH 3T3 pBABE-CK2α cell line, which stably express CK2α, was created by retroviral delivery and grown in the presence of 2 μg/ml puromycin, as previously described [9]. NIH 3T3 cell lines stably expressing pBABE-GFP was grown under the same conditions, and harvested at the same cell confluency (75-90%) and used as control.

Plasmids and transfection analyses. The expression vectors pCDNA3-FLAG-IKKi (IKKi) and pCDNA3-FLAG-IKKi K38A (IKKi K38A) encode wild type and kinase inactive forms of human IKKi, respectively (kind gift of T. Maniatis, Harvard University, Cambridge MA [24]). The pRC/CMV-HA-CK2α', pRC/CMV-HA CK2α K68A and pRC/CMV-myc-CK2β vectors were generously provided by D. Litchfield (University of Western Ontario, Canada [32]). The pcDNA3-FLAG-IKKα or pcDNA3-FLAG-IKKβ vectors expressing IKKα and IKKβ, respectively have been described elsewhere[33]. To evaluate NF-κB activity, wild type (WT) and mutant NF-κB element-thymidine kinase promoter-chloramphenical acetyl transferase (CAT) reporter vectors, containing two WT NF-κB elements (E8-CAT) or two mutated NF-κB elements (mut-E8-CAT) from upstream of the *c-myc* promoter [4] or an NF-κB-element drive luciferase reporter construct, kindly provided by G. Rawadi (Hoechst-Marion-Roussel, Romainville, France) [34] were used. NF-κB promoter constructs used include: 1) *cyclin D1* promoter: containing WT (-66 wt-Luc) or mutant (-66 mut-Luc) NF-κB elements (kind gift of R.G. Pestell, Lombardi Comprehensive Cancer Center, Washington DC) [29, 35]; 2) *relB* promoter: containing WT (-1.7 κB) or mutant (-1.7 κB mut) versions of the two NF-κB elements, prepared as described previously [36]. For transfection into six well plates, 4 μg total DNA was transfected per well. When using p100 tissue culture plates, 10 μg of DNA was transfected per plate. For transient transfection into Hs578T cells, cells were incubated for 16-24 hours with DNA and GenePorter2 (Gene Therapy Systems), according to the manufacturer's directions. For MMTV-Her2/*neu* NF639 cell lines,

transfections were performed by incubating for 16 hours in the presence of DNA and Fugene 6 Transfection Reagent (Roche) according to the manufacturer's directions. The calcium phosphate method of transient transfection [37] was used with HEK293T cells, and cultures harvested 48 hours post transfection. CAT and luciferase assays were performed as described previously in [5] and [29], respectively. Co-transfection of an SV40-β-galactosidase (SV40-β-gal) expression vector was used to normalize for transfection efficiency, as described previously [8]. Significance was determined using the Student t-test.

Immunoblotting. Cytoplasmic protein extracts prepared in homogenization buffer (10 mM HEPES [pH 7.9], 100 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM sucrose, 10 mM NaF, 10 mM β-glycerophosphate, p-nitrophenyl phosphate, 1 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin). Cytoplasmic extracts islolated from tissue culture treated cells were prepared in RSB buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 10 mM NaF, 10 mM β-glycerophosphate, p-nitrophenyl phosphate, 1 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin) and nuclear extracts were prepared in DR buffer (20 mM HEPES [pH 7.9], 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol). Protein concentration was measured by Bradford assay using the Bio-Rad reagent (Bio-Rad), according to the manufacturer's directions. Cytoplasmic protein extracts (30 μg) were separated using 10% polyacrylamide (PAGEN-terminal IKKi (sc-9913), C-terminal IKKi (sc-5694), IκB-α (sc-203), IκB-β (sc-945), and HA (sc-805) were purchased from Santa Cruz. Antibodies against IκB-ε and c-Myc were a gift from N. Rice and M. Ernst (NCI, Fredrick MD) and S. Hann ((Vanderbilt University, Nashville TN), respectively. Competition assays for IKKi were performed using 2 μg cognate peptide (sc-9913 P). Antibodies against cyclin D1 (06-137), and glutathione S-transferase (GST) (27-4577-01) were purchased from Upstate Biotechnology, and Pharmingen, respectively. A monoclonal FLAG antibody derived from the M5 epitope (Sigma) was used to detect N-terminally FLAG-tagged proteins. To normalize for loading, blots were stripped and reprobed with a β-actin specific antibody (AC-15, Sigma). Quantitation by scanning densitometry was performed with a KDS1D device (version 2.0; Kodak).

IKKi kinase assay. To prepare whole cell extracts (WCEs), cells were washed with PBS, resuspended in cold kinase assay lysis buffer [20 mM Tris (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 250 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM dithiothreitol, and 0.25% Nonidet-P40] and lysed by sonication. Cellular debris was removed by centrifugation and extracts were precleared with protein A/G agarose Plus beads (Santa Cruz) and bovine anti-goat HRP secondary antibody (Santa Cruz) for 1 hour at 4°C. In the case of HEK293T cells, preclearing was performed with protein A/G agarose Plus beads, and bovine anti-mouse HRP secondary antibody (Santa Cruz) for 1 hour at 4°C. IKKi complexes were isolated from a 500 μl reaction mixture containing 500 μg of proteins in the aforementioned buffer with 2 μg of antibody against IKKi (sc-9913 - as defined above) for endogenous IKKi or with an antibody against the M5 epitope of FLAG for transiently transfected HEK293T cells expression FLAG-IKKi. The 500 μl samples in PD buffer were split into three aliquots. One third was used for a kinase assay with GST-IκB-α as a substrate, and

one third for a kinase assay with GST- 2N-IκB-α with mutated serine residues at position 32 and 36 (to assess specificity of IKKi). Alternatively, GST-IκB-β was used as a substrate. All kinase assays were performed at 30°C for 45 minutes in the presence of 3 μCi γ-^{32}P ATP and 10 μM unlabeled ATP. The kinase reactions were stopped by the addition of PAGE-SDS sample buffer, subject to 10% PAGE-SDS and visualized by autoradiography. The remaining one-third of immunoprecipitated proteins were subject to immunoblot analysis for IKKi using an antibody (sc-5694, C-terminal, K-14) with an epitope derived from a different peptide sequence of the protein.

RT-PCR. RNA was isolated using Trizol, according to the manufacturer's protocol (Invitrogen). Total RNA was quantified by measuring A_{260} and only samples with an A_{260}:A_{280} ratio of between 1.8 and 2 used. To remove contaminating DNA, RNA samples were digested for 30 min at 37°C with RQ1 RNase-free DNase (Promega Corporation), according to the manufacturer's directions. RNA was re-extracted and ethanol precipitated. For reverse transcriptase (RT)-PCR, 1 μg RNA samples were reverse transcribed with Superscript RNase H-RT in the presence of 150 ng of oligo-dT and 100 ng of random primers (all reagents from Invitrogen). PCR were performed in a Thermal Cycler (MJ Research) by denaturing at 94°C for 60 sec, annealing at 57°C for 45 sec and extending at 72°C for 55 sec for 32 cycles. The primer pairs designed using the Primer Designer software program (Scientific and Education Software). The pairs specific for human IKKi, which amplified a 472 bp product, were (Forward, nucleic acid position 504 bp) 5'-CGGAAGCTGAACCACCAGAA-3' and (Reverse, 976 bp) 5'-CCAGTGGCTGCATGGTACAA-3'. To amplify mouse IKKi, the following mouse specific primer pairs were used (Forward, 1465 bp) 5'-CTTCAACTCAGCCAGCTATC-3' and (Reverse, 1892 bp) 5'-GGTGCAGGTATTCCTCTGTA-3' (which amplified a 427 bp product). The β-actin primers were (Forward, 516) 5'-CACTGGCATCGTGATGGACT-3' and (Reverse, 923) 5'-CGGATGTCCACGTCACACTT-3' (which amplified a 407 bp product). Quantitation was performed by scanning densitometry as above.

RESULTS

IKKi is expressed in human breast cancer primary specimens and cell lines. To determine whether human breast tumors express IKKi, cytoplasmic extracts from six breast cancer tissue specimens were assessed by immunoblot analysis for IKKi expression. A band of the appropriate molecular weight for IKKi (80 kDa) was seen in 4 of the 6 samples (Fig. 1A, left panel). Analysis of β-actin levels confirmed that loading was essentially equivalent. To verify the specificity of the IKKi bands detected in these tumor samples, a duplicate blot was subjected to immunoblotting in the presence of 2 μg of cognate peptide, as competitor (Fig. 1A, right panel). Detection of the 80 kDa band was completely eliminated with the addition of the cognate peptide. The antibody also recognized several lower molecular weight bands, which were also eliminated upon addition of the cognate peptide; these likely represent IKKi degradation products (data not shown). These findings indicate that IKKi is indeed expressed in multiple human primary breast cancer specimens.

To further investigate the expression of IKKi in human mammary epithelial and breast cancer cells, the MCF-10F, D3-1 and Hs578T cell lines were selected for

investigation. The D3-1 cell line was derived from the immortalized untransformed MCF-10F cells by DMBA-mediated transformation [30]. The Hs578T breast cancer cell line was derived from a carcinosarcoma, and is epithelial in origin. Immunoblotting of whole cell extracts was performed using an N-terminal antibody followed showed substantially higher levels of IKKi in Hs578T and D3-1 cells than in MCF-10F cells (Fig. 1B). (Similar data were obtained using immunoprecipitation of IKKi followed by immunoblotting, see Fig. 1B below.) To measure IKKi kinase activity, immunoprecipitated IKKi from the various lines were subjected to a kinase assay using as substrate either GST-WT-IκB-α or a mutant GST-2N-IκB-α protein, which confirmed the specificity of the assay (Fig. 1C). Of the two transformed cell lines, Hs578T exhibited a higher level of IKKi activity compared to the D3-1 cells; although, they appeared to contain essentially equal levels of protein as judged by immunoblotting (Fig. 1A and 1B). No IKKi activity was detectable in the MCF-10F cells in this or a duplicate experiment (Fig. 1B). Importantly, the two transformed cell lines display higher levels of NF-κB activity compared to the untransformed MCF-10F cells [8], consistent with the higher IKKi activity.

IKKi expression is elevated in mammary tissue and tumors of MMTV-CK2α and MMTV CK2α x c-*rel* bitransgenic mice. Since patient samples and human breast cancer cell lines showed expression of IKKi, we next sought to determine whether mouse mammary tumors display elevated levels of IKKi, and selected the following mouse models: MMTV-CK2α, expressing the CK2α catalytic subunit, and the MMTV-c-*rel*, expressing the c-Rel NF-κB subunit. Approximately 30% of female MMTV-CK2α transgenic mice developed a variety of mammary tumors at a median age of 23 months [22], while 31.6% of female MMTV-c-*rel* mouse developed one or more tumors at an average age of 19.9 months [29]. Cytoplasmic extracts from mammary glands of age and pregnancy matched wild type (WT) mice were compared to those from tumors and histologically normal mammary glands of transgenic MMTV-CK2α (Fig. 2A) and MMTV-c-*rel* mice (Fig. 2B). Expression of IKKi protein was extremely low in the extracts from mammary glands of pregnant WT animals (Figs. 2A and 2B). The MMTV-CK2α mouse tumor (7367T) displayed substantially higher levels than the WT-1 and WT-2 mouse mammary gland (Fig. 2A), while 4 of 5 tumors derived from MMTV-c-*rel* displayed a higher level of IKKi protein compared to the mammary glands of these two WT mice (Fig. 2B). As control for protein loading, the gel was stained with Coomassie, which indicated essentially equal loading. Interestingly, we noted that the histologically normal mammary glands of the MMTV-CK2α mice all appeared to contain substantial levels of IKKi as compared to the mammary glands of WT animals, whereas barely detectable levels were present in the histologically normal glands of the MMTV-c-*rel* transgenic mouse. Thus, the mammary glands of MMTV-CK2α animals appear to express higher levels of IKKi than do the MMTV-c-*rel* mice, which led us to test the potential role of CK2 in the enhanced expression.

We have recently prepared a bitransgenic MMTV-CK2 x MMTV-c-*rel* mice, which exhibited a 77% incidence of breast tumor formation with the average age at onset of 20.8 months (S. Guo, unpublished observations). If CK2 promotes IKKi levels, then the histologically normal mammary glands of the bitransgenic mice would display elevated levels of IKKi. Histologically normal mammary glands of bitransgenic MMTV-CK2α x MMTV-c-*rel* mice were removed from the mice that developed tumors and the

levels of IKKi protein compared in 3 animals (Fig. 2C). Substantial IKKi expression was detected in cytoplasmic extracts of mammary glands from these mice. The tumors from the same animal displayed somewhat higher IKKi levels. These findings are similar to the data obtained with the MMTV-CK2α mice, and support a role for CK2 in the induction of IKKi expression. Consistent with this hypothesis, previously we showed that the levels of CK2 expression were highest in Hs578T cells, followed by D3-1 and then MCF-10F cells [8]. Taken together, these data indicate that mouse and human breast cancer tissue display elevated IKKi protein expression.

To begin to elucidate the mechanism of IKKi activation, RT-PCR was performed using RNA isolated from a histologically normal mammary gland and a tumor from mouse 5885 mammary glands of age. GAPDH mRNA expression levels were used to normalize. As can be seen from the RT-PCR results (Fig. 2C, right panel), mRNA levels of IKKi are higher in the tumor compared to histologically normal, consistent with the protein expression of IKKi. This suggests that CK2 plays a role in up-regulation of IKKi at the mRNA level and that this effect may be enhanced in mammary tumors compared to mammary gland tissue.

Transient CK2 transfection elevates IKKi levels in HEK293T and NIH 3T3 cells. The tetrameric CK2 enzyme is composed of two catalytic subunits CK2α or CK2α', and two CK2β regulatory subunits. To test whether CK2 can directly increase IKKi levels, cultures of HEK293T cells were transfected with vectors expressing either the CK2α' subunit as a HA-tagged protein (pRc/CMV-HA-CK2α') or the regulatory subunit CK2β as a c-Myc-epitope tagged protein (pRc/CMV-myc-CK2β), or a combination of both vectors. WCEs were prepared and samples subjected to immunoblotting for IKKi, and for the expression of the transfected CK2 subunits (Fig. 3A). Immunoblotting for HA or c-Myc confirmed the expression of the CK2α' and CK2β subunit, respectively. Expression of either subunit alone or in combination led to a substantial increase in IKKi levels. Analysis of β-actin confirmed equal sample loading. To confirm that CK2 acted at a pre-translational level to increase IKKi expression, semi-quantitative RT-PCR was performed using total RNA isolated from cells transfected as above (Fig. 3B). As a positive control, cells were transfected with a vector expressing IKKi. Ectopic expression of either CK2 subunit led to a substantial induction of IKKi mRNA levels. Analysis of β-actin was used to normalize loading, and verify RNA quality. The data from two independent experiments were quantified, normalized to β-actin, and the mean values presented relative to empty vector pCDNA3 control in the lower panel (Fig. 3B). Thus, transient ectopic overexpression of CK2 leads to induction of IKKi at both the mRNA and protein level in HEK293T cells.

To investigate the ability of CK2α to increase IKKi levels, we analyzed two clones of NIH 3T3 cells stably expressing CK2α (termed Clone 5 and Clone 6) compared to parental pBABE DNA as control. These lines, which were isolated previously through retroviral infection and puromycin selection, display elevated CK2 activity [9]. Higher levels of IKKi were seen in WCEs of Clone 5 and Clone 6 as compared with the clones infected with the pBABE-GFP control viral vector (Fig. 3C). Analysis of β-actin confirmed equal sample loading. Thus, overexpression of CK2α leads to induction of IKKi protein in NIH 3T3 cells.

Inhibition of CK2 activity decreases IKKi levels in breast cancer cells. To determine whether inhibition of CK2 would lead to a reduction in IKKi expression, Hs578T breast

cancer cells were selected since they display high levels of activity of CK2 (Romieu-Mourez et al., 2001) and IKKi (see Fig. 1C above). Hs578T cells were incubated for 6 h in the presence of either 25 or 50 μM apigenin, a selective inhibitor of CK2 activity, or with an equal volume of carrier DMSO. RNA was isolated and subjected to semi-quantitative RT-PCR (Fig. 4A). As compared to control cells treated with DMSO, a decrease in IKKi mRNA expression was noted at 25 μM apigenin (2.5-fold) and a further decrease in mRNA levels were seen at the 50 μM concentration (2.9-fold) (Fig. 4a). Because 50 μM dose of apigenin, showed the greater effect on IKKi mRNA levels, it was the dose selected to test the effects on IKKi protein. Treatment of Hs578T cells with 50 μM apigenin for 6 hours resulted in a substantial decrease in IKKi protein levels (Fig. 4B). Thus, inhibition of CK2α with a selective pharmacologic mediator leads to decreased IKKi mRNA expression.

Since pharmacologic inhibitors may also affect other kinases, a vector expressing kinase-inactive CK2α subunit, which functions as a competitive inhibitor with endogenous CK2α, was used [9, 32]. Hs578T breast cancer cells were stably transfected with a vector expressing HA-tagged kinase inactive CK2α (K68M) or an empty vector control DNA, and CEs prepared. Hs578T CK2α (K68M) stable expressing cells displayed lower levels of IKKi compared to empty vector control cells (Fig. 4C). Immunoblotting for HA confirmed the ectopic HA-CK2α (K68A) expression. Taken together, these findings indicate that CK2 activity induces expression of IKKi.

Ectopic IKKi induces the degradation of IκB-α and increases NF-κB activity in HEK293T cells and kinase inactive IKKi increases IκB-α stability in Hs578T breast cancer cells. Previous studies demonstrated involvement of IKKi phosphorylates IκB-α on Ser36 [25, 38]. We next tested the kinase activity of IKKi on IκB-α, and IκB-β NF-κB inhibitory proteins, using GST-fusion forms as substrates. HEK293T cells, which express only a low endogenous level of IKKi (Fig. 3B), were co-transfected in duplicate with either pcDNA3-FLAG-IKKi expression vector or control pCDNA3 empty vector DNA [38]. As additional controls, cells were transfected with the pcDNA3-FLAG-IKKα or pcDNA3-FLAG-IKKβ vectors expressing IKKα and IKKβ, respectively. WCEs were immunoprecipitated using a monoclonal FLAG antibody, and subjected to a kinase assays with the appropriate IκB protein substrate (Fig. 5). Ectopic expression of IKKi resulted in a clear increase in activity with either IκB-α-GST (55 kDa) or IκB-β-GST (55 kDa) as substrate compared to the control. As a positive control for immunoprecipitation of FLAG fusion proteins, FLAG-tagged IKKα and IKKβ were similarly transfected, immunoprecipitated and subjected to kinase assays using IκB-α as substrate. Thus, IKKi can phosphorylate IκB-α and IκB-β *in vitro*.

The effects of IKKi on the steady state levels of IκB-α, IκB -β and IκB-ε in HEK293T cells were determined next. Cytoplasmic extracts were prepared 48 hours post-transfection with either pcDNA3-FLAG-IKKi expression vector or control pcDNA3 empty vector DNA, and assessed by immunoblotting of levels of IκB expression, and β-actin, which confirmed essentially equal protein loading. Ectopic IKKi expression caused a dramatic reduction in the total amount of cytoplasmic IκB-α (Fig. 5B). Only a modest drop in IκB-β levels was detected and no effect was seen on levels of IκB-ε. Overall, ectopic IKKi expression appears to selectively modulate stability of IκB-α protein in HEK293T cells.

Previous studies demonstrated IKKi induces NF-κB activity [24, 25]. To confirm the ability of IKKi to induces functional NF-κB activity in HEK293T cells, cultures were transiently transfected with a 2-copy NF-κB element TK-CAT reporter construct (termed E8-CAT) in the presence of either pcDNA3-FLAG-IKKi vector expressing IKKi or pcDNA3 empty vector DNA and an SV-40 β-gal vector to normalize for transfection efficiency. A 3.2-fold induction of the reporter construct was seen when HEK293T cells were transfected with pcDNA3-FLAG-IKKi compared to those transfected with the parental pcDNA3 vector (Fig. 5C, left panel). Similarly, transient co-transfection of pcDNA3-FLAG-IKKi into HEK293T cells with a synthetic 5-copy κB-luciferase construct (termed κB-luc.) resulted in a substantial increase in reporter activity compared to cells transfected with control pcDNA3 DNA. This effect was further enhanced by the addition of PMA (100 ng/ml) for 18 hours before measuring reporter activity (Fig. 5C, right panel) as has been reported previously [24]. Thus, consistent with previous findings [24, 25], IKKi induces functional NF-κB activity which is promoted by PMA. The increase in NF-kB activity appears to be directly related to the ability of IKKi to phosphorylate IκB-α at serine 36 promoting its subsequent degradation.

Kinase inactive IKKi K38A increases IκB-α protein and reduces NF-κB-driven reporter activity in Hs578T breast cancer cells. To assess the functional role of IKKi in the constitutive NF-κB activity detected in breast cancer cells, a kinase inactive IKKi (IKKi K38A) with a mutation in the activation loop that blocks activity and serves as dominant negative form was used. The effects of an increasing dose of the IKKi K38A expression on WT vs mutant NF-κB element driven promoter activity was assessed in Hs578T breast cancer cells using either E8-CAT or mut-E8-CAT, which is driven by two mutant NF-κB elements (Fig. 6). Expression of the kinase inactive IKKi resulted in a dose-dependent decrease in NF-κB activity in Hs578T breast cancer cells. In particular, a 25% reduction of E8-CAT NF-κB-driven activity at the lowest dose of 1 μg IKKi K38A ($p<0.05$), and a nearly 5-fold reduction of E8-CAT activity was seen at 2 μg of IKKi K38A ($p<0.005$) (Fig. 6A). At the highest dose of kinase inactive IKKi, the activity of the WT NF-κB E8-CAT was reduced almost to that of the reporter driven by the mutant elements mut-E8-CAT. We next sought to determine whether the inhibitory effects of IKKi activity in Hs578T cells could be related to changes in IκB-α levels. The remaining extracts were subjected to immunoblot analysis. We first confirmed the expression of IKKi K38A by using a monoclonal FLAG-specific antibody. An increasing level of IKKi was seen with increasing dose of IKKi K38A vector, as expected. To examine steady-state levels of IκB proteins, the blots were probed with antibodies against either IκB-α or IκB-β. A dramatic increase in the levels of IκB-α was observed with increasing amounts of IKKi K38A, while no change in IκB-β levels was observed (Fig. 6B). Immunoblot analysis of β-actin levels confirmed equal loading. These results suggest that the regulation of NF-κB activity by IKKi results from changes in the levels of IκB-α, which is a target of IKKi in breast cancer cells.

To verify that the functional role of IKKi in regulation of NF-κB was not cell type specific, NF639 cells were selected since CK2 activity in these cells had previously been implicated in the induction of NF-κB (Romieu-Mourez, 2002). Immunoblotting confirmed the presence of active IKKi protein (data not shown). Cultures of NF639 cells transfected with either pCDNA3 or IKKi K38A expression vectors in the presence of

either E8-CAT or mut-E8-CAT NF-κB reporter constructs. The remaining extracts were probed for IKKi expression to confirm that IKKi K38A was being expressed. Significant repression of the E8 reporter was observed. Thus, inhibition of IKKi in these mammary cancer cells reduces NF-κB activity, suggesting this kinase plays a more general role in the aberrant activation of NF-κB in breast cancer.

IKKi K38A reduces activity of NF-κB-driven natural promoters *cyclin D1* and *relB* in Hs578T breast cancer cells. To test the effects of the kinase inactive IKKi on natural promoters driven by NF-κB elements, co-transfection analysis was performed using the *cyclin D1* and *relB* promoters, which are each driven by two NF-κB elements [35, 36]. Co-transfection of the IKKi K38A vector in Hs578T breast cancer cells resulted in a dose-dependent decrease in activity of the wild type *cyclin D1*-luciferase reporter construct (-66 wt-Luc), whereas the mutant –66 mut-Luc was largely unaffected (Fig. 7A). To verify IKKi K38A reduced cyclin D1 protein levels, p100 plates were transfected with 10 μg of pcDNA3 or 10 μg of pcDNA3-IKKi K38A. 48 hours post transfection, WCEs were isolated and subject to immunoblotting for cyclin D1, which showed significant suppression when transfected with IKKi K38A. β-actin confirmed equal loading. Similarly co-transfection of IKKi K38A with p1.7 *relB*-Luc *relB* promoter-reporter construct, containing two WT NF-κB sites, reduced luciferase activity to levels seen with a mutant p1.7 *relB*-Luc construct, which contains mutations in the two identified NF-κB elements (Fig. 7B. left panel). Lastly, to test whether the endogenous genes are affected, Hs578T cells were transiently transfected with IKKi K38A using GenePorter2, which provides a transfection efficiency of ~20-30% with these cells (data not shown). Nuclear and cytoplasmic extracts were prepared and subjected to immunoblot analysis using a RelB antibody, which confirmed a reduction in nuclear RelB expression (Fig. 7B, right panel). Similar results were obtained when we assessed these extracts for cyclin D1. The blots were stripped and reprobed for β-actin, which confirmed equal loading. Thus, taken together these studies provide strong evidence that IKKi plays an important role in control of NF-κB activity in breast cancer cells.

CONCLUSIONS AND DISCUSSION

Here, we demonstrate for the first time the functional role of IKKi in the aberrant activation of NF-κB in breast cancer and implicate CK2 in the induction of IKKi mRNA and protein levels. IKKi expression was detected in primary human breast tumor specimens and cell lines, and mammary tumors from MMTV-CK2α, MMTV-c-*rel*, and MMTV-CK2α x MMTV-c-*rel* bitransgenic mice. Importantly, we noted that histologically normal mammary glands of MMTV-CK2α and MMTV-CK2α x MMTV-c-*rel* bitransgenic mice also displayed elevated levels of IKKi compared to samples from age-matched wild type mice, suggesting a role for CK2 activity in regulation of IKKi. Ectopic expression of CK2 subunits in cells induced IKKi levels. Conversely inhibition of CK2 with the selective CK2 inhibitor apigenin or upon expression of a kinase inactive subunit in Hs578T breast cancer lines reduced endogenous IKKi levels. Previous studies from our lab have shown that a hallmark of breast cancer is the aberrant activation of NF-κB, which promotes tumor cell survival, growth and transformed phenotype [5, 9, 39]. The data presented here implicate the induction of IKKi by CK2 as a new signaling pathway in this activation, and identify IKKi as a potential new chemotherapeutic target.

The LPS inducible kinase, IKKi was first identified as a murine IKK-like protein that displayed high expression in the spleen with approximately 30% amino acid identity to IKKα and IKKβ in the kinase domain [25]. Subsequently, the human form of IKKi was also identified as having high expression in the spleen and was cloned and characterized in Jurkat T cells [24] showing a marked increase in activity, but not expression, upon treatment with the PKC activator PMA. Interestingly, a lack of strict correlation was noted in the level of detectable protein compared to activity of IKKi in D3-1 and Hs578T cells, suggesting the possible role of additional activation steps. These data clearly indicated that IKKi played a central role in immune response, however a precise biological role was not forthcoming. Because the similarity between IKKi and IKKα and IKKβ, it was first tested for its ability to phosphorylate IκB-α using *in vitro* kinase assays. It was found to specifically phosphorylate serine 36 of IκB-α and not serine 32. It was first believed that these kinases make up part of an alternate IKK complex that lead to NF-κB/Rel activation, however recent studies in IKKi$^{-/-}$ MEFs [40], have shown that LPS treatment continues to activate NF-κB/Rel activation suggesting that the activation normally medicated by the toll-like receptor, implicated in LPS signaling, occurs independent of IKKi. This, however, does not rule out other signaling events from occurring. Our data shows that IKKi does indeed play a role in NF-κB activation in breast cancer, suggesting that its function may be cell-type dependent, signal specific or responsible for partial NF-κB/Rel activation.

Previous studies have shown that IKKi phosphorylates IκB-α *in vitro*, and that induction of the kinase leads to NF-κB reporter activation. Our results confirm the ability of IKKi to phosphorylate IκB-α *in vitro*, and demonstrate its ability to regulate IκB-α levels and NF-κB activity in cells in culture. Furthermore, while our studies indicate IKKi can also phosphorylate IκB-β in vitro, the steady state levels of IκB-β (and those of IκB-ε) were not affected by transient transfection with IKKi in HEK293T cells. Thus, our findings suggest IκB-α is a preferential *in vivo* target of IKKi. Consistent with previous work [24], we observed that the inhibition by IKKi K38A led to a decrease in activities of

the *cyclin D1* and *relB* promoters, which are driven by NF-κB elements, essentially down to baseline levels.

Previous studies from our lab have shown that TGF-β1 treatment reduces NF-κB/Rel activity in breast cancer cells [39]. It has also been demonstrated that TGF-β1 treatment of NMuMG normal murine mammary epithelial cells leads to a detectable suppression of basal IKKi mRNA expression by 1 hour, and to a nearly complete suppression between 6-24 hours [41]. Consistent with our data presented in this manuscript, hepatocytes treated with TGF-β1 block activity of CK2 and stabilize IκB-α levels [42]. Breast cancer cell lines and tumor specimens have been shown to express elevated levels of CK2 [8, 9, 22, 23, 42]. Thus, taken together, these results suggest that TGF-β1 treatment reduces CK2 activity, which can have a direct effect on IκB-α stability and may also suppress IKKi expression further enhancing stability of IκB-α and blocking subsequent NF-κB/Rel activation. Overall, our studies identify for the first time a role for IKKi in NF-κB/Rel activation in breast cancer and identify its potential role as a theraputic target in the treatment of breast cancer.

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Figure Legends

FIG. 1. IKKi is expressed in breast cancer cells. **A)** Cytoplasmic extracts were prepared from the indicated human breast tumor specimen and samples (50 μg) used to prepare duplicate blots. These were subjected to immunoblotting with 2 μg of antibody specific for IKKi (Q-15, sc-9913) in the absence or presence of excess [2 μg] cognate IKKi peptide as competitor. One blot was stripped and reprobed with an antibody against β-actin to confirm loading. **B)** Whole cell extracts (50 μg) were prepared from human mammary epithelial cells and subjected to immunoblotting for IKKi expression. **C)** Whole cell extracts (WCEs) were prepared in PD buffer and precleared with Protein A/G Agarose beads and anti-goat secondary IgG HRP conjugated antibody. IKKi complexes were immunoprecipitated from 300 μg of protein using an N-terminal derived antibody. One third was used for a kinase assay on a GST-WT-IκB-α as substrate (KA), one third for a kinase assay on a GST-2N-IκB-α as substrate (KA) and one third for western blotting for IKKi (K-14, sc-5694, a C-terminal derived antibody) (IB).

FIG. 2. IKKi expression in mouse tumor models. Cytoplasmic extracts were prepared from mammary glands of age-matched wild type (WT) mice and histologically normal mammary glands from transgenic mice (N), and transgenic mouse tumor(s) (T), as indicated: A) MMTV-CK2α transgenic mice; B) MMTV-c-*rel* transgenic mice; C) (left panel) MMTV-CK2α X MMTV-c-*rel* bi-transgenic mice. Samples (50 μg) were subjected to immunoblotting for IKKi expression. Coomassie blue staining of duplicate gels was performed to confirm essentially equal loading. C) cDNA was prepared from RNA islolated from MMTV-CK2 X MMTV-c-rel bi trasnsgenic mice and a histologically normal sample and a tumor sample from mouse 5885 was used to determine IKKi expression levels. GAPDH confirmed equal loading.

FIG. 3. IKKi is induced by ectopic CK2 expression in HEK293T and NIH 3T3 cells. **A)** Cultures of HEK293T cells (in 6 well P-60 dishes) were transfected in triplicate with 1 μg of pRC/CMV-HA-CK2α' or pRC/CMV-myc-CK2β DNA alone or in combination and enough pCDNA3 (CMV driven) vector to make a total of 2 μg DNA. Samples of cytoplasmic extracts (50 μg) were subjected to immunoblot analysis for IKKi expression. To confirm expression of CK2α' and CK2β the blot was stripped and re-probed with antibodies specific for HA and c-Myc, respectively. Equal loading was confirmed by probing the blot with a β-actin antibody. **B)** HEK293T cells were transfected as above with pRC/CMV-HA-CK2α' or pRC/CMV-myc-CK2β DNA alone or in combination, or with 2 μg of pCDNA3-FLAG-IKKi expressing FLAG-tagged IKKi, as a positive control. RNA was prepared using Trizol reagent, and digested with DNase. Samples (1 μg) were used for first strand cDNA synthesis, and a 2 μl aliquot (total 50 μl) used to perform RT-PCR analysis for IKKi and β-actin. Two independent experiments were performed, quantified, normalized to β-actin, and the mean level of expression relative to the control pCDNA3 DNA for the two experiments is given. **C)** Clones of NIH 3T3 cells infected with either pBABE-CK2α retrovirus expressing CK2α or a parental pBABE-GFP vector were isolated as described previously [9]. Samples of whole cell extract (50 μg) were subjected to immunoblotting for either IKKi or β-actin, which confirmed equal loading.

FIG. 4. IKKi suppression in epithelial breast cancer cells trough inhibition of CK2. **A** Hs578T cells were grown in 6 well plates and treated with 25 μM or 50 μM for 6 hours. RNA was harvested using Trizol reagent and DNase treated. 1 μg of DNase-treated RNA was used for first strand synthesis and a 2 μl aliquot was used for RT-PCR analysis of IKKi expression. RT-PCR for β-actin was performed to confirm that the starting material was of the same concentration. The experiment was performed in duplicate with identical results. **B** Hs578T breast cancer cells were treated with apigenin for 6 hours at a concentration of 50 μM. Whole cell extract was prepared and 50 μg was electrophoresed, blotted and probed for IKKi expression. Blots were stripped and reprobed with a β-actin antibody to confirm equal loading. **C** Hs578T cells were transfected with either 2 μg of empty parental vector or 2 μg of a kinase inactive construct of CK2α (pRc/CMV-HA-CK2α K68M, labeled CK2α KI). Cells were harvested and collected 16 hours post-transfection, washed with PBS and lysed in RIPA buffer. Immunoblots were prepared with 50 μg of whole cell extract and probed for IKKi expression. Confirmation of the kinase inactive CK2α was confirmed by probing with a CK2α antibody and equal loading was confirmed by probing the blot with an antibody for β-actin.

FIG. 5. IKKi phosphorylates IκB-α and IκB-β *in vitro* and overexpression of IKKi increases IκB-α turnover and NF-κB activity *in vivo*. **A)** HEK293T cells were plated in p100 dishes and transfected the following day with 10 μg of pcDNA3, pcDNA3-FLAG-IKKi, pcDNA3-FLAG-IKKα or pcDNA-FLAG-IKKβ. 48 hours post transfection, cells were harvested and lysed in PD buffer and 150 μg was subject to immunopurification using a monoclonal FLAG antibody (Sigma). Immunocomplexes were precipitated using Protein A/G agarose (Santa Cruz). One half of the purified immunocomplexes were subject to kinase assays with γ-^{32}P-ATP using 200 ng of GST- IκB-α and IκB-β as substrates. Transfections and reactions were performed in duplicate. FLAG tagged IKKα and IKKβ were immunoprecipitated with the monoclonal FLAG antibody using GST-IκB-α as a substrate for positive controls. Negative controls consisting of immunoprecipitated protein extract from empty vector transfected cells, substrate and γ-^{32}P-ATP were also run. **B)** HEK293T cells were transfected triplicate with 2 μg of empty parental vector or pcDNA3-FLAG-IKKi. 48 hours post transfection, cells were harvested and cytoplasmic extracts prepared. 20 μg of cytoplasmic extract was electrophoresed, blotted and probed for IκB-α, IκB-β and IκB-ε expression. Odd numbered lanes represent empty vector transfected and even numbered lanes represent pcDNA-FLAG-IKKi transfected HEK293T cells. Blots were stripped and probed with β-actin to control for equal loading. A control blot was probed with a monoclonal FLAG antibody detecting FLAG-IKKi fusion protein. **C)** NF-κB activity in response to transient IKKi expression in HEK293T cells. HEK293T cells were co-transfected in duplicate with 2 μg of E8-CAT reporter construct, 0.5 μg of SV40-β-gal and either 2.5 μg of pCDNA3 or 2.5 μg of pCDNA3-FLAG-IKKi. CAT activity was measured and normalized to β-gal levels. Alternatively to test NF-κB reporter activity, cells were transfected in triplicate with 1 μg κB-luciferase reporter construct, 0.5 μg SV40-β-gal and 2 μg of pCDNA3 or 2 μg of pCDNA3-FLAG-IKKi. One day post transfection, cells were treated with 100 ng/ml

PMA and harvested 18 hours later when luciferase activities were determined and normalized to β-gal activity levels.

FIG. 6. Inhibition of IKKi activity in breast cancer cell blocks NF-κB activity and increases the levels of IκB-α. **A)** NF-κB E8-CAT reporter activity in Hs578T cells is reduced by dn-IKKi in a dose-dependent manner. Hs578T cells were co-transfected, in triplicate, with either 2 μg of E8-CAT reporter construct or 1 μg of mut-E8-CAT along with 1 μg of SV40-β-gal, to normalize for transfection efficiency, and increasing concentrations of pCDNA3-FLAG-IKKi K38A [0-2 μg] while maintaining a 4 μg total DNA concentration with parental pCDNA3 vector. Whole cell extracts (WCEs) were prepared, normalized for transfection efficiency and subjected to CAT assays. Values for the mean +/- SD are presented. Significance was determined using the Student t-test. **B)** IKKi K38A leads to an increase in steady state levels of IκB-α in Hs578T breast cancer cells. Whole cell extracts (20 μg) from above were subjected to immunoblot analysis for the FLAG epitope, to confirm IKKi expression. The blots were stripped and re-probed with an antibody for IκB-α, IκB-β and subsequently for β-actin, which confirmed equal loading. **C)** NF-κB E8-CAT reporter activity in MMTV-Her2/*neu* NF639 breast cancer cells is reduced by dn-IKKi. MMTV-Her2/*neu* NF639 cells were co-transfected in triplicate with 2 μg of pCDNA3 parental vector or pcDNA3-FLAG-IKKi K38A, 0.5 μg E8-CAT reporter construct and 0.5 μg of SV40-β-gal for normalization of transfection efficiency. Western blotting for IKKi confirmed that IKKi K38A transfected NF639 cells expressed higher levels of IKKi compared to EV controls.

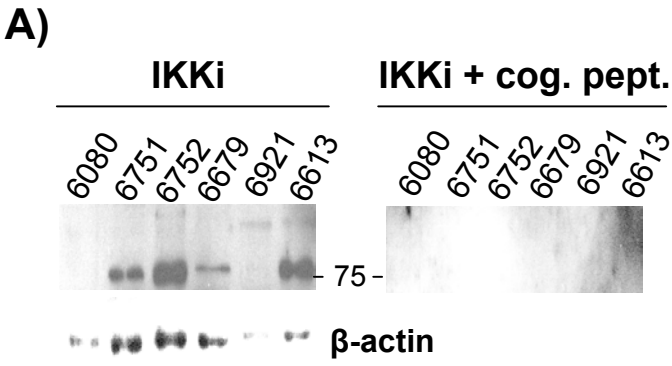
FIG. 7 IKKi K38A suppresses *cyclin D1* and *relB* promoter activities and expression in Hs578T breast cancer cells **A)** Cyclin D1 luciferase reporter activity is blocked by dn-IKKi. Left panel, Hs578T cells were co-transfected, in triplicate, with either 1.0 μg *cyclin D1* –66 luciferase or *cyclin D1* mut-66 luciferase reporter construct along with 1.0 μg of SV40-β-gal and increasing concentrations of pCDNA3-FLAG-IKKi K38A (0-2.0 μg) while maintaining a 4 μg total DNA concentration with parental pCDNA3 vector. WCEs, were subjected to luciferase assays and normalized for transfection efficiency. Values for the mean +/- SD are presented. Significance was determined using the Student t-test. Right panel, Hs578T breast cancer cells were plated in p100 plates and transfected with 10 μg of pCDNA3-FLAG-IKKi K38A, WCEs were prepared and 30 μg WCE was electrophoresed and subjected to immunoblotting for cyclin D1 expression. **B)** *relB* luciferase reporter activity is inhibited by IKKi K38A. Left panel, Hs578T cells were co-transfected, in duplicate, with either 1.0 μg –1.7 kb *relB* luciferase or –1.7 kB mut-*relB* luciferase reporter construct along with 1.0 μg of SV40-β-gal and 2.0 μg of pCDNA3 or 2.0 μg of pCDNA3-FLAG-IKKi K38A. Results are typical of two independently performed experiments. WCEs, normalized for transfection efficiency, were subjected to luciferase assays. Values for the mean +/- SD are presented. Significance was determined using the Student t-test. Right panel, IKKi expression reduces cytoplasmic and nuclear levels of RelB in Hs578T breast cancer cells. Hs578T breast cancer cells were plated in p100 plates and transfected with 10 μg of pCDNA3-FLAG-IKKi K38A. Cytoplasmic and nuclear extracts were isolated, and samples (20 μg and 10 μg, respectively) subjected to immunoblotting for RelB and cyclin D1 expression.

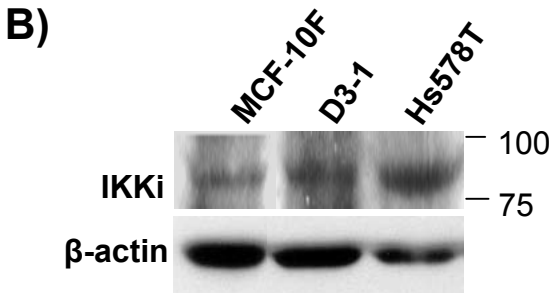
References

1. Siebenlist, U., G. Franzoso, and K. Brown, *Structure, regulation and function of NF-kappa B.* Annu Rev Cell Biol, 1994. **10**: p. 405-55.
2. Rayet, B. and C. Gelinas, *Aberrant rel/nfkb genes and activity in human cancer.* Oncogene, 1999. **18**(49): p. 6938-47.
3. Smith, M.R. and W.C. Greene, *Identification of HTLV-I tax trans-activator mutants exhibiting novel transcriptional phenotypes.* Genes Dev, 1990. **4**(11): p. 1875-85.
4. Duyao, M.P., A.J. Buckler, and G.E. Sonenshein, *Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter.* Proc Natl Acad Sci U S A, 1990. **87**(12): p. 4727-31.
5. Sovak, M.A., et al., *Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer.* J Clin Invest, 1997. **100**(12): p. 2952-60.
6. Nakshatri, H., et al., *Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth.* Mol Cell Biol, 1997. **17**(7): p. 3629-39.
7. Kim, D.W., et al., *Activation of NF-kappaB/Rel occurs early during neoplastic transformation of mammary cells.* Carcinogenesis, 2000. **21**(5): p. 871-9.
8. Romieu-Mourez, R., et al., *Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-kappaB in breast cancer.* Cancer Res, 2001. **61**(9): p. 3810-8.
9. Romieu-Mourez, R., et al., *Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells.* Cancer Res, 2002. **62**(22): p. 6770-8.
10. Yamaoka, S., et al., *Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation.* Cell, 1998. **93**(7): p. 1231-40.
11. Rothwarf, D.M., et al., *IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex.* Nature, 1998. **395**(6699): p. 297-300.
12. Schwarz, E.M., D. Van Antwerp, and I.M. Verma, *Constitutive phosphorylation of IkappaBalpha by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IkappaBalpha.* Mol Cell Biol, 1996. **16**(7): p. 3554-9.
13. McElhinny, J.A., et al., *Casein kinase II phosphorylates I kappa B alpha at S-283, S-289, S-293, and T-291 and is required for its degradation.* Mol Cell Biol, 1996. **16**(3): p. 899-906.
14. Barroga, C.F., et al., *Constitutive phosphorylation of I kappa B alpha by casein kinase II.* Proc Natl Acad Sci U S A, 1995. **92**(17): p. 7637-41.
15. Arsura, M., M. Wu, and G.E. Sonenshein, *TGF beta 1 inhibits NF-kappa B/Rel activity inducing apoptosis of B cells: transcriptional activation of I kappa B alpha.* Immunity, 1996. **5**(1): p. 31-40.
16. Guerra, B. and O.G. Issinger, *Protein kinase CK2 and its role in cellular proliferation, development and pathology.* Electrophoresis, 1999. **20**(2): p. 391-408.

17. Allende, J.E. and C.C. Allende, *Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation.* Faseb J, 1995. **9**(5): p. 313-23.
18. Keller, D.M., et al., *A DNA damage-induced p53 serine 392 kinase complex contains CK2, hSpt16, and SSRP1.* Mol Cell, 2001. **7**(2): p. 283-92.
19. Kato, T., Jr., et al., *CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response.* Mol Cell, 2003. **12**(4): p. 829-39.
20. Pando, M.P. and I.M. Verma, *Signal-dependent and -independent degradation of free and NF-kappa B-bound IkappaBalpha.* J Biol Chem, 2000. **275**(28): p. 21278-86.
21. Miyamoto, S., B.J. Seufzer, and S.D. Shumway, *Novel IkappaB alpha proteolytic pathway in WEHI231 immature B cells.* Mol Cell Biol, 1998. **18**(1): p. 19-29.
22. Landesman-Bollag, E., et al., *Protein kinase CK2 in mammary gland tumorigenesis.* Oncogene, 2001. **20**(25): p. 3247-57.
23. Landesman-Bollag, E., et al., *Protein kinase CK2: signaling and tumorigenesis in the mammary gland.* Mol Cell Biochem, 2001. **227**(1-2): p. 153-65.
24. Peters, R.T., S.M. Liao, and T. Maniatis, *IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex.* Mol Cell, 2000. **5**(3): p. 513-22.
25. Shimada, T., et al., *IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IkappaB kinases.* Int Immunol, 1999. **11**(8): p. 1357-62.
26. Nomura, F., et al., *NF-kappaB activation through IKK-i-dependent I-TRAF/TANK phosphorylation.* Genes Cells, 2000. **5**(3): p. 191-202.
27. Pomerantz, J.L. and D. Baltimore, *NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase.* Embo J, 1999. **18**(23): p. 6694-704.
28. Bonnard, M., et al., *Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription.* Embo J, 2000. **19**(18): p. 4976-85.
29. Romieu-Mourez, R., et al., *Mouse mammary tumor virus c-rel transgenic mice develop mammary tumors.* Mol Cell Biol, 2003. **23**(16): p. 5738-54.
30. Calaf, G. and J. Russo, *Transformation of human breast epithelial cells by chemical carcinogens.* Carcinogenesis, 1993. **14**(3): p. 483-92.
31. Elson, A. and P. Leder, *Protein-tyrosine phosphatase epsilon. An isoform specifically expressed in mouse mammary tumors initiated by v-Ha-ras OR neu.* J Biol Chem, 1995. **270**(44): p. 26116-22.
32. Penner, C.G., Z. Wang, and D.W. Litchfield, *Expression and localization of epitope-tagged protein kinase CK2.* J Cell Biochem, 1997. **64**(4): p. 525-37.
33. Mercurio, F., et al., *IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation.* Science, 1997. **278**(5339): p. 860-6.
34. Rawadi, G., et al., *Involvement of small GTPases in Mycoplasma fermentans membrane lipoproteins-mediated activation of macrophages.* J Biol Chem, 1999. **274**(43): p. 30794-8.
35. Guttridge, D.C., et al., *NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1.* Mol Cell Biol, 1999. **19**(8): p. 5785-99.

36. Wang, X. and G.E. Sonenshein, *Induction of the RelB NF-kappaB subunit by the cytomegalovirus IE1 protein is mediated via Jun kinase and c-Jun/Fra-2 AP-1 complexes.* J Virol, 2005. **79**(1): p. 95-105.
37. Chen, C. and H. Okayama, *High-efficiency transformation of mammalian cells by plasmid DNA.* Mol Cell Biol, 1987. **7**(8): p. 2745-52.
38. Peters, R.T. and T. Maniatis, *A new family of IKK-related kinases may function as I kappa B kinase kinases.* Biochim Biophys Acta, 2001. **1471**(2): p. M57-62.
39. Sovak, M.A., et al., *The inhibitory effects of transforming growth factor beta1 on breast cancer cell proliferation are mediated through regulation of aberrant nuclear factor-kappaB/Rel expression.* Cell Growth Differ, 1999. **10**(8): p. 537-44.
40. Kravchenko, V.V., et al., *IKKi/IKKepsilon plays a key role in integrating signals induced by pro-inflammatory stimuli.* J Biol Chem, 2003. **278**(29): p. 26612-9.
41. Xie, L., et al., *Transforming growth factor beta-regulated gene expression in a mouse mammary gland epithelial cell line.* Breast Cancer Res, 2003. **5**(6): p. R187-98.
42. Cavin, L.G., et al., *Inhibition of CK2 activity by TGF-beta1 promotes IkappaB-alpha protein stabilization and apoptosis of immortalized hepatocytes.* Hepatology, 2003. **38**(6): p. 1540-51.


A)
IKKi
IKKi + cog. pept.
6080
6751
6752
6679
6921
6613
75
β-actin


B)
MCF-10F
D3-1
Hs578T
100
75
IKKi
β-actin

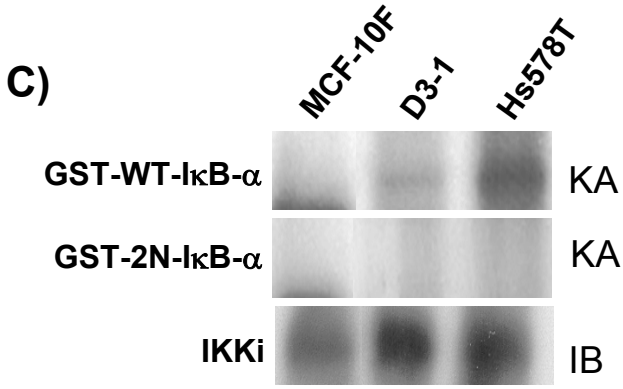
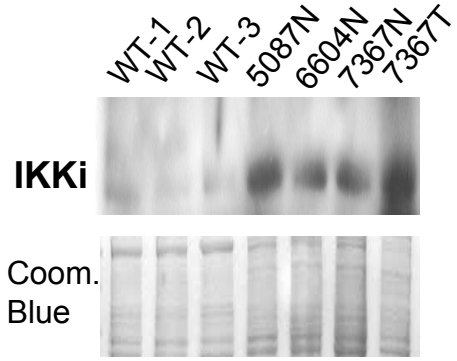
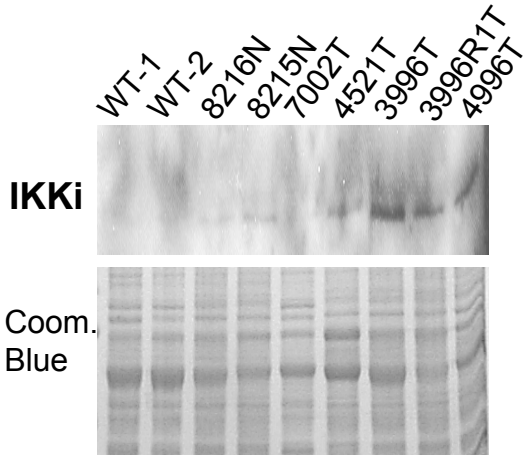

C)
MCF-10F
D3-1
Hs578T
GST-WT-IκB-α
KA
GST-2N-IκB-α
KA
IKKi
IB

Figure 1

A) MMTV-CK2 transgenic mice



B) MMTV-*c-rel* transgenic mice



C) MMTV-CK2 X MMTV-c-*rel* bi-transgenic

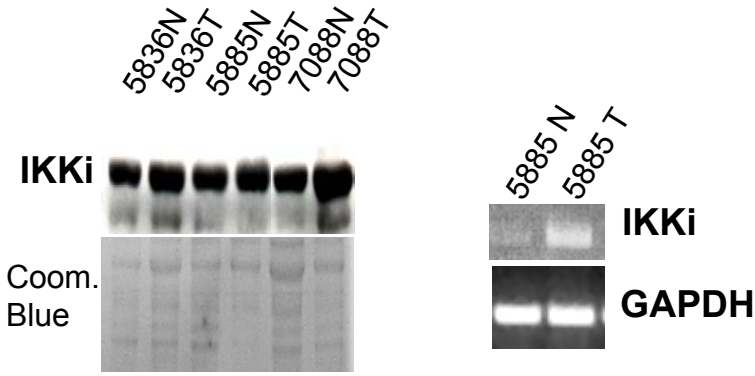


Figure 2

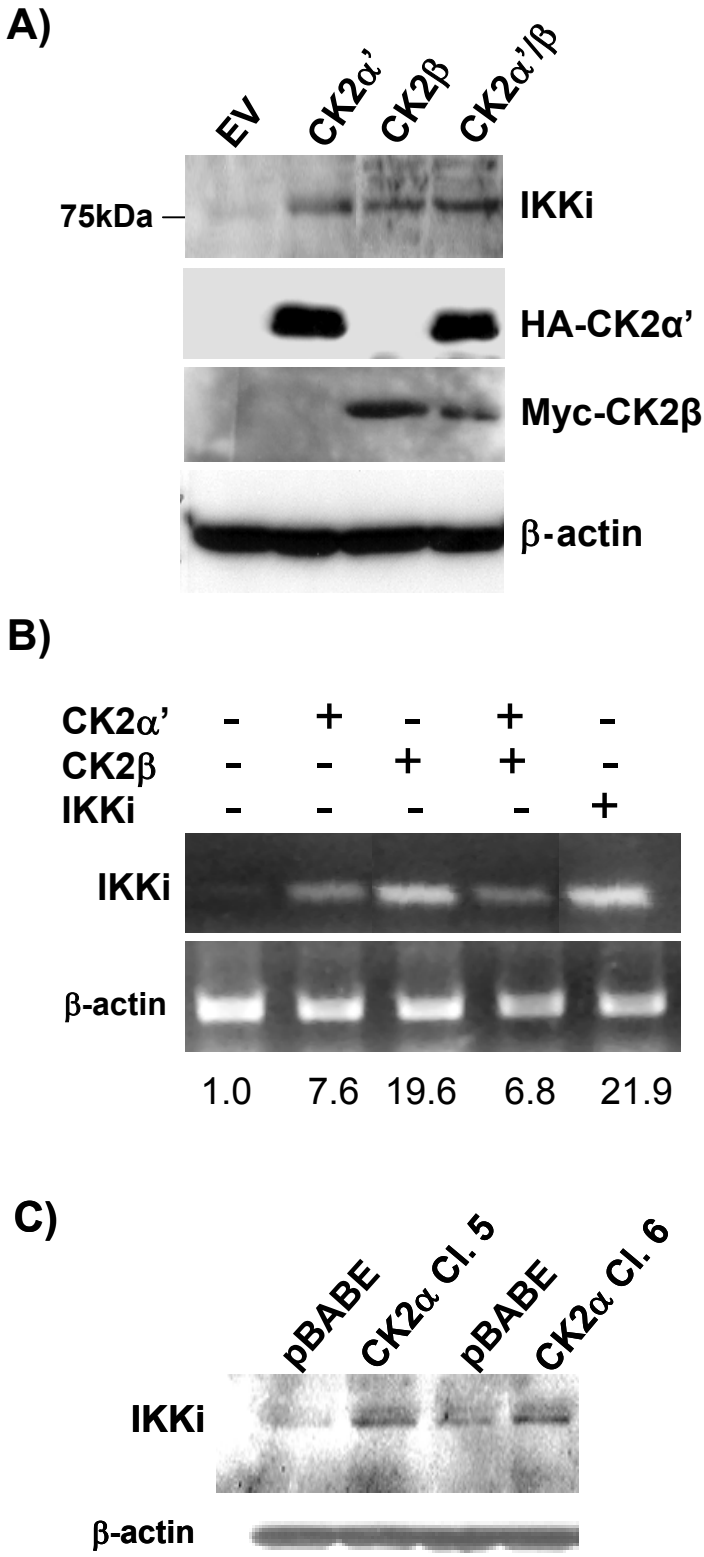

A)
EV
CK2α'
CK2β
CK2α'/β
75kDa
IKKi
HA-CK2α'
Myc-CK2β
β-actin
B)
CK2α' - + - + -
CK2β - - + + -
IKKi - - - - +
IKKi
β-actin
1.0 7.6 19.6 6.8 21.9
C)
pBABE
CK2α Cl. 5
pBABE
CK2α Cl. 6
IKKi
β-actin

Figure 3

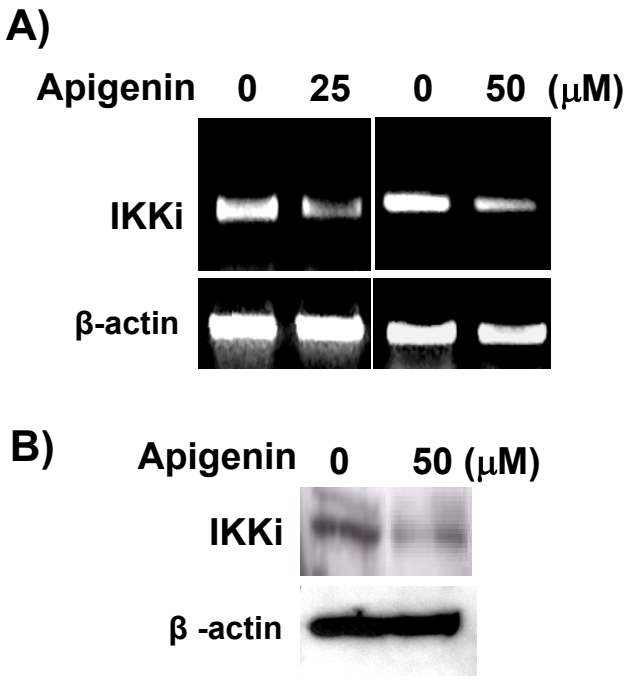
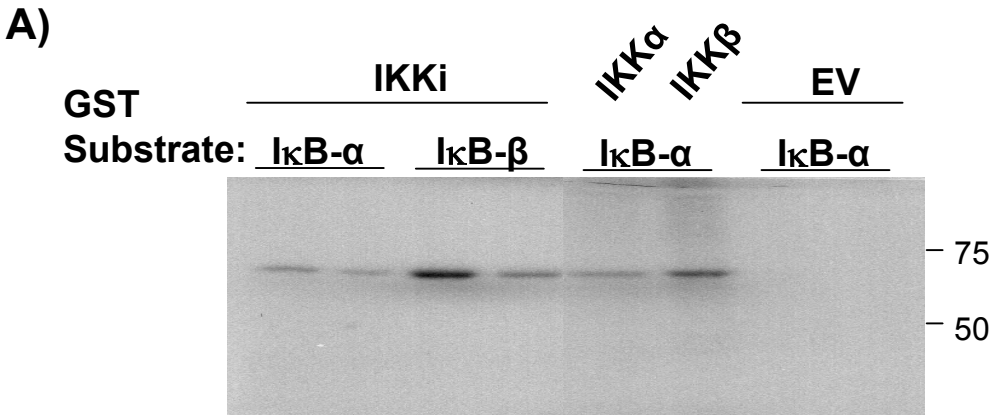
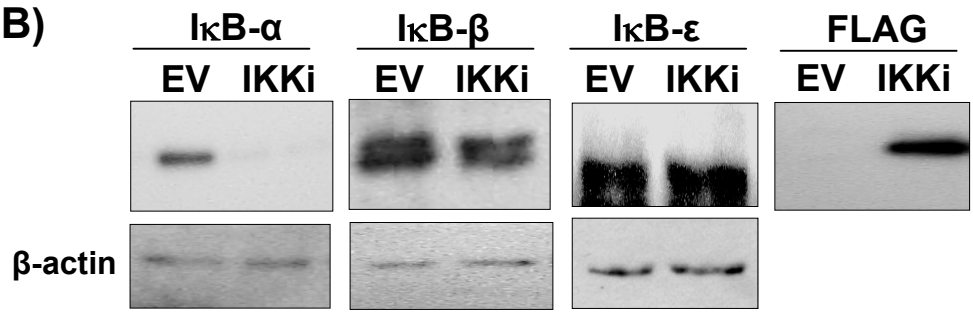

A)
Apigenin 0 25 0 50 (μM)
IKKi
β-actin
B)
Apigenin 0 50 (μM)
IKKi
β -actin
C)
EV
KI
IKKi
HA
β-actin

Figure 4


A)
GST
Substrate:
IKKi
IKKα
IKKβ
EV
IκB-α
IκB-β
IκB-α
IκB-α
75
50


B)
IκB-α
IκB-β
IκB-ε
FLAG
EV
IKKi
EV
IKKi
EV
IKKi
EV
IKKi
β-actin

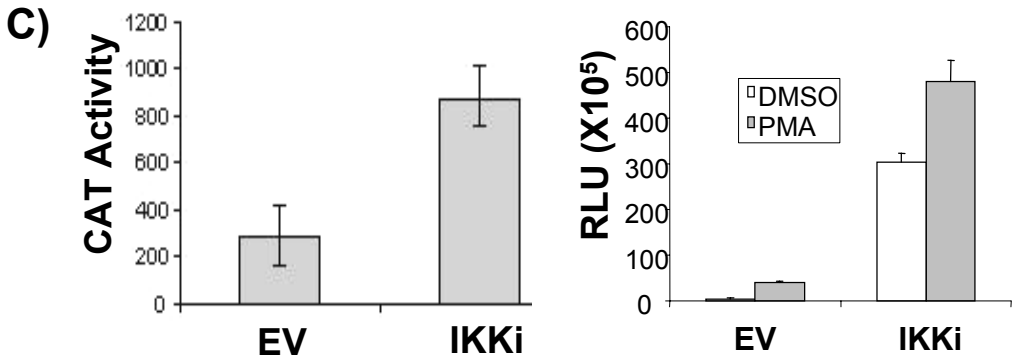

C)
CAT Activity
1200
1000
800
600
400
200
0
EV
IKKi
RLU (X10⁵)
600
500
400
300
200
100
0
DMSO
PMA
EV
IKKi

Figure 5

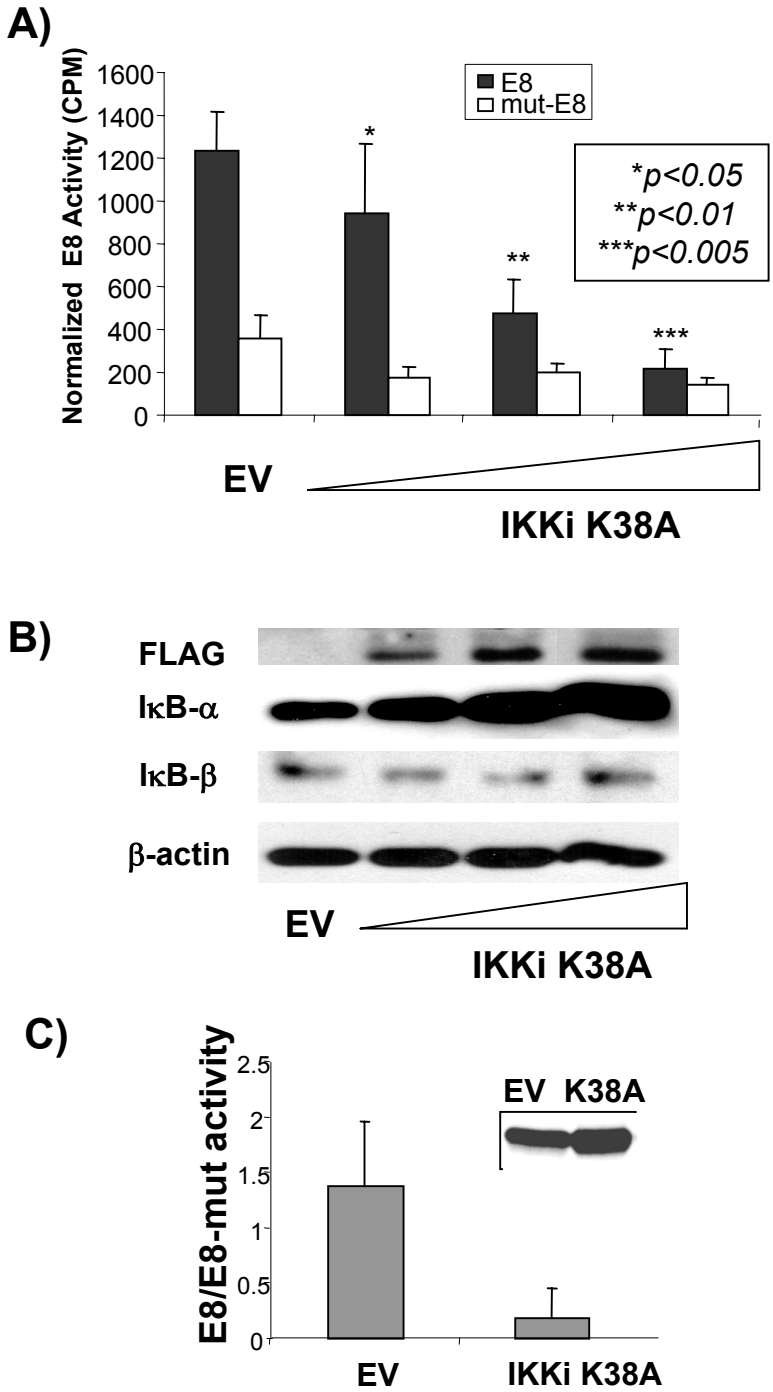
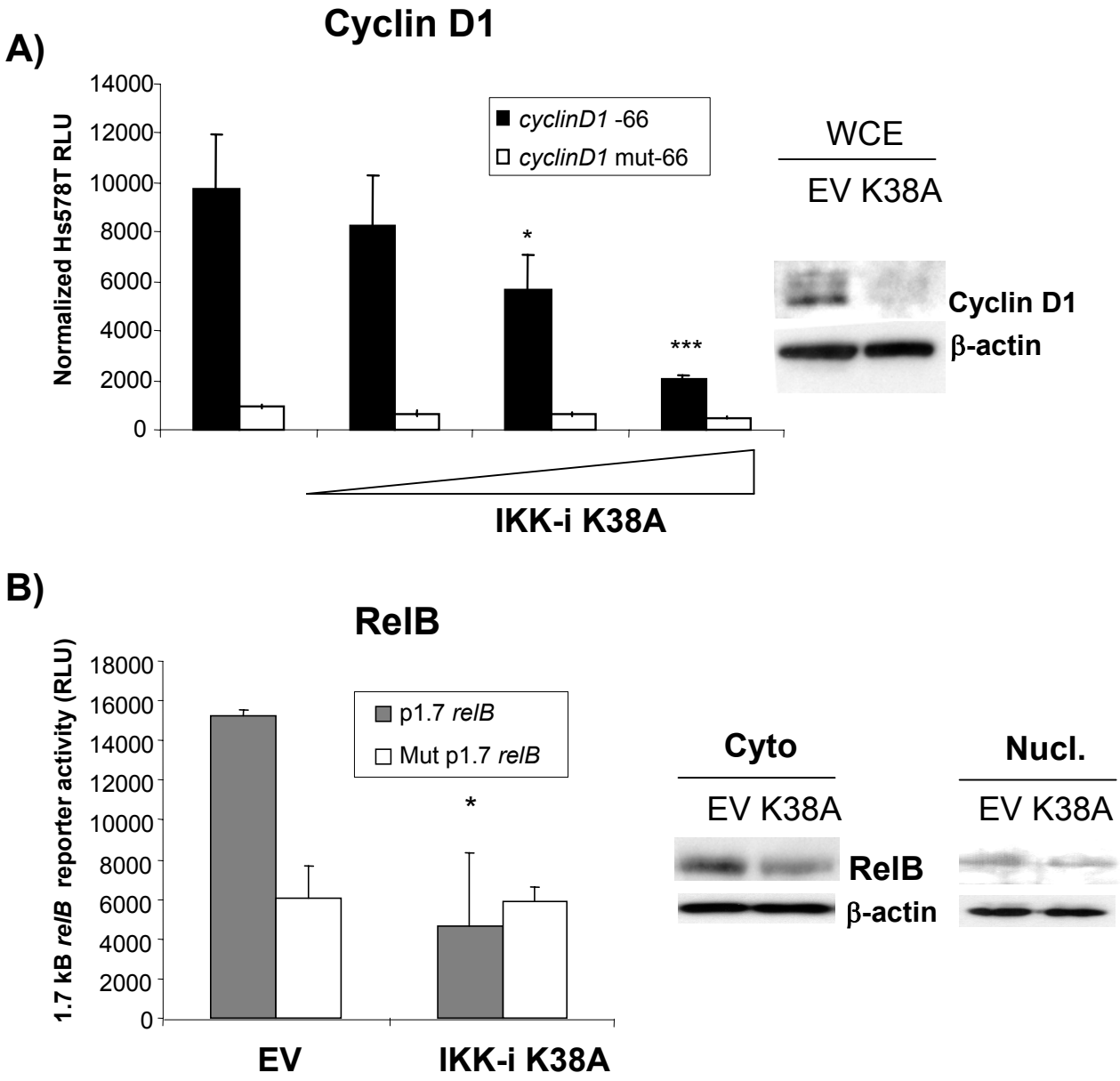

A)
Normalized E8 Activity (CPM)
1600
1400
1200
1000
800
600
400
200
0
E8
mut-E8
*
**
***
*p<0.05
**p<0.01
***p<0.005
EV
IKKi K38A
B)
FLAG
IκB-α
IκB-β
β-actin
EV
IKKi K38A
C)
E8/E8-mut activity
2.5
2
1.5
1
0.5
0
EV K38A
EV
IKKi K38A

Figure 6


A)
Cyclin D1
Normalized Hs578T RLU
14000
12000
10000
8000
6000
4000
2000
0
cyclinD1 -66
cyclinD1 mut-66
*
***
WCE
EV K38A
Cyclin D1
β-actin
IKK-i K38A
B)
RelB
1.7 kB relB reporter activity (RLU)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
p1.7 relB
Mut p1.7 relB
*
Cyto
Nucl.
EV K38A
EV K38A
RelB
β-actin
EV
IKK-i K38A

KEY RESEARCH ACCOMPLISHMENTS : REPORTABLE OUTCOMES

Manuscripts

Eddy, S. F., Guo, S., Demicco, E., Landesman-Bollag, E., Seldin, D.C. and Sonenshein, G.E. CK2 Induces IKKi/IKKε in Breast Cancer Cells. *In press* Cancer Research.

Romieu-Mourez R. Landesman-Bollag E., Seldin DC., Sonenshein GE. "Protein kinase CK2 promotes aberrant activation of NF-κB, transformed phenotype and survival of breast cancer cells" (2002) Cancer Res., **62** 6770-6778.

Romieu-Mourez, R., Kim, D.W., Shin, S.M., Demicco, E.G., Landesman-Bollag, E., Seldin, D.C., Cardiff, R.D. and Sonenshein, G.E. (2003) Mouse mammary tumor virus c-rel transgenic mice develop mammary tumors. Mol Cell Biol. **23,** 5738-5754.

Abstracts

Sean F. Eddy, S.G. Guo, C.M. Taylor, E. Landesman-Bollag, D.E. Seldin and G.E. Sonenshein. CK2 induces IKK-i/IKKε in breast cancer cells. Era of Hope Department of Defense Breast Cancer Research Program Meeting. Philadelphia, PA. June 8-11, 2005.

Romieu-Mourez R., . Kim DW., Shin S., Landesman-Bollag E., Seldin DC., and Sonenshein GE. "c-Rel or activation of NF-kB by protein kinase CK2 promote breast cancer" Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Sep. 25-28, 2002, Orlando, Florida (poster presentation).

APPENDIX

Please find attached reprints of manuscripts published under DAMD 17-01-1-0158:

Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D.C., Sonenshein, G.E. Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells. Cancer Res., 2002 62: 6770-6778.

Romieu-Mourez, R., Kim, D.W., Shin, S.M., Demicco, E.G., Landesman-Bollag, E., Seldin, D.C., Cardiff, R.D., Sonenshein, G.E. Mouse mammary tumor virus c-*rel* transgenic mice develop mammary tumors. Mol. Cell. Biol. 2003, 23: 5738-5754.

Eddy, S.F., Guo, S., Demicco, E.G., Landesman-Bollag, E., Seldin, D.C., Sonenshein, G.E. IKK-i/IKKε Expression is Induced by CK2 and Promotes Aberrant NF-κB Activation in Breast Cancer Cells

Please also find attached reprints of manuscripts complementing Aims 1-5:

Landesman-Bollag, E., Romieu-Mourez, R., Song, D.H., Sonenshein, G.E., Cardiff, R.D., Seldin, D.C. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene, 2001, 20: 3247-3257.

Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D.C., Traish, A.M., Mercurio, F., Sonenshein, G.E. Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-kappaB in breast cancer. Cancer Res. 2001, 61 : 3810-3818.

[CANCER RESEARCH 62, 6770–6778, November 15, 2002]

Protein Kinase CK2 Promotes Aberrant Activation of Nuclear Factor-κB, Transformed Phenotype, and Survival of Breast Cancer Cells[1]

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ABSTRACT

The *Her-2/neu* oncogene, the second member of the epidermal growth factor (EGF) receptor family, encodes a transmembrane tyrosine kinase receptor. Overexpression of Her-2/neu in ~30% of breast cancers is associated with poor overall survival. Recently, we have found that Her-2/neu activates nuclear factor (NF)-κB via a phosphatidylinositol 3 kinase (PI3-K)-Akt kinase signaling pathway in mouse mammary tumor virus (MMTV)-Her-2/neu NF639 mouse breast cancer cells. Surprisingly, the IκB kinase (IKK) kinase complex, implicated in proteasome-mediated degradation of IκB-α and activation of NF-κB via the canonical pathway, was not activated in these cells. Degradation of IκB-α was mediated via calpain, which in B cells is facilitated by phosphorylation of IκB-α by the protein kinase CK2. Here, we report that the inhibition of CK2 blocks Her-2/neu-mediated activation of NF-κB. NF639 breast cancer cells, stably expressing CK2α or CK2α′ kinase-inactive mutants, displayed decreased NF-κB binding and reduced ability to grow in soft agar, as well as increased sensitivity to tumor necrosis factor (TNF)-α killing. Similarly, CK2 kinase-inactive subunits inhibited NF-κB activity in Hs578T human breast cancer cells, which also display elevated CK2 activity. In NIH 3T3 fibroblasts, which express low basal NF-κB and CK2 activities, overexpression of CK2 by retroviral gene delivery led to increased IκB-α turnover and the induction of classical NF-κB (p50/RelA). Thus, CK2 plays an important role in Her-2/neu signaling, promoting IκB-α degradation and, thereby, NF-κB activation. Furthermore, because ectopic CK2 activity appears sufficient to induce NF-κB, the elevated CK2 activity observed in many primary human breast cancers likely plays a role in aberrant activation of NF-κB and, therefore, represents a potential therapeutic target.

INTRODUCTION

NF-κB[3]/Rel is a family of dimeric transcription factors distinguished by the presence of a 300-amino-acid region, termed the Rel homology domain (1). The NF-κB family includes five known members in mammals: p50/p105, p52/p100, c-Rel, RelB, and RelA (p65). Classical NF-κB complexes are composed of p50/RelA heterodimers. In most untransformed cells, other than B lymphocytes, NF-κB/Rel proteins are sequestered in the cytoplasm bound to the specific IκB-inhibitory proteins, of which IκB-α is the paradigm. A variety of agents that activate NF-κB, *e.g.*, TNF-α and interleukin 1, mediate degradation of IκB-α via a canonical pathway involving phosphorylation of IκB on two NH_2-terminal serine residues by a large multisubunit complex composed of the two IKKs IKKα and IKKβ (2, 3). This phosphorylation is followed by ubiquitination and rapid proteasome-mediated degradation of IκB, allowing for translocation of free NF-κB to the nucleus. In addition, phosphorylation of IκB by protein kinase CK2 (formerly casein kinase II) has been implicated in basal IκB-α degradation (4–8). CK2-mediated phosphorylation of IκB-α occurs preferentially at Ser-283, Ser-289, Thr-291, and Ser-293 within the COOH-terminal PEST domain, and mutations of these sites prolong IκB-α half-life (5, 7, 8). In lymphoid cells, an alternative calpain-mediated IκB-α degradation pathway was described that contributed to the constitutive NF-κB activation seen in these cells (9). Moreover, we demonstrated that CK2 phosphorylation accelerates calpain-mediated degradation of IκB in B cells (10). Thus, two alternative phosphorylation signals and proteolytic systems regulate degradation of IκB and activation of NF-κB in what appears to be a signal-and cell type-specific manner.

CK2 is a highly conserved serine/threonine kinase that recognizes the general consensus sequence (S/T)XX(D/E). CK2 is a constitutively active kinase that is ubiquitously expressed in both the cytoplasm and nucleus of eukaryotic cells and exists in cells as a holoenzyme containing two catalytic (CK2α or CK2α′) and two regulatory (CK2β) subunits. The two catalytic subunits are highly homologous, but CK2α′ has a unique required role in spermatogenesis (11). The crystal structure of human $CK2\alpha_2\beta_2$ was recently solved, showing flexible interdomain and intersubunit interactions in which each catalytic subunit makes no direct contact with the other and each interacts with both regulatory subunits, via the NH_2-terminal lobe of the catalytic subunit and an extended COOH-terminal tail of the regulatory subunit (12). CK2 is essential for cell viability, and many of the >160 CK2 substrates identified thus far are growth- and cell cycle-related (13). Overexpression or inhibition of CK2α, CK2α′, or CK2β subunits was shown to affect proliferation; however, results varied greatly with cell type (14–17). Overexpression of CK2 has been observed in many human cancers, including breast cancers (18–21). Furthermore, we have shown that enforced CK2α expression in transgenic mice is sufficient to induce T-cell lymphomas (22) and breast cancer (19).

We and others have demonstrated aberrant activation of NF-κB factors in breast cancer (23–27). High levels of nuclear NF-κB/Rel were found in human breast tumor cell lines and in the majority of primary human and rodent breast tumor tissue samples. In contrast, untransformed breast epithelial cells and normal mammary glands contain low basal levels of nuclear NF-κB/Rel. In breast cancer cells, elevated levels of NF-κB correlated with a decrease in the half-life of IκB-α protein (25). We observed that many primary breast tumor tissue samples and human cancer cells display an increase in either CK2 or IKK activity (20).

Recently, we have found that the Her-2/neu protein activates NF-κB via a PI3-K- to Akt kinase-signaling pathway that can be inhibited via the tumor suppressor PTEN (28). The *Her-2/neu* (or c-*erbB-2*) oncogene, the second member of the EGF receptor family (EGFR-2), encodes a transmembrane tyrosine kinase receptor. Overexpression of Her-2/neu, which is seen in ~30% of breast cancers, is associated with poor overall survival, increased metastatic potential and resistance to chemotherapeutic agents (29). Surprisingly, degra-

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[2] To whom requests for reprints should be addressed, at Department of Biochemistry, Boston University Medical School, 715 Albany Street, Boston, MA 02118-2394. E-mail: gsonensh@bu.edu.

[3] The abbreviations used are: NF-κB, nuclear factor-κB; β-gal, β-galactosidase; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift analysis; GST, glutathione-*S*-transferase; PI3-K, phosphatidylinositol 3-kinase; WCE, whole cell extract; WT, wild type; MMTV, mouse mammary tumor virus; IKK, IκB kinase; TNF, tumor necrosis factor; CMV, cytomegalovirus; Oct1, Octomer-1 (oligonucleotide); PEST, proline-glutamic-serine-threonine rich region.

dation of IκB-α in the MMTV-Her-2/neu-derived mammary tumor cells NF639 (30) did not appear to be mediated via the IKK complex or the proteasome but, rather, was blocked on addition of calpain inhibitors (28). This raised the question of involvement of CK2 in activation of NF-κB in NF639 cells, which we have addressed here. Expression of kinase-inactive CK2 catalytic subunit mutants decreased basal NF-κB activity in NF639 cells, as well as in Hs578T human breast cancer cells, which were shown to overexpress CK2 (20). Furthermore, the inhibition of CK2 enhanced susceptibility to cell death and inhibited transformed phenotype of NF639 cells. Conversely, ectopic CK2 was sufficient to induce NF-κB activity in NIH 3T3 fibroblasts, which express low basal NF-κB and CK2 activities. These studies indicate that CK2 directly controls NF-κB activity, which, in turn, modulates the survival and transformed phenotype of breast cancers, including those in which *Her-2/neu* is amplified.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions. The NF639 MMTV-c-neu mouse mammary tumor cell line (kindly provided by P. Leder, Harvard Medical School, Boston, MA) was cultured as described previously (30). NMuMG is an untransformed, immortalized mouse mammary epithelial cell line and was cultured as described previously (31). The Hs578T human breast tumor cell line, which was derived from a carcinosarcoma and is epithelial in origin, was grown as described previously (24). NIH 3T3 mouse fibroblast, 293T human embryonic kidney cell line, and Phoenix packaging cell lines were cultured in DMEM supplemented with 10% FCS and penicillin-streptomycin (Invitrogen Life Technologies, Inc., Carlsbad, CA). For protein synthesis inhibition, exponentially growing cells were treated with 30–50 μg/ml cycloheximide (Sigma-Genosys Inc., The Woodlands, TX).

Transfection Conditions. For transfections, Fugene reagent was used according to the manufacturer's directions (Roche Diagnostics Corporation, Indianapolis, IN). To evaluate transcriptional activity, cells were transfected in triplicate with an NF-κB element-luciferase reporter vector, driven by three κB elements from upstream of the MHC class I promoter, kindly provided by A. Chan (Mt. Sinai School of Medicine, New York City, NY; Ref. 32). The SV40-promoter β-gal (pSV40-β-gal) reporter vector was used to normalize transfection efficiency. Luciferase assays were performed as described previously (33). Statistical analyses were performed using the Student's *t* test. The pSVK3-human IκB-α WT, 2N (S32A and S36A), 3C (S283A, T291A and T299A), and 2N3C (S32A, S36A, S283A, T291A, and T299A) plasmid vectors were a kind gift from J. Hiscott (Institut Lady Davis de Recherches Medicales; Ref. 5). The pRc/CMV2 HA-tagged CK2α (pZW2), pRc/CMV2-HA-CK2α K68M (pGP8), pRc/CMV2-HA-CK2α′ (pGP3), and pRc/CMV2-HA-CK2α′ K69M (pGP18) vectors, and the backbone empty vector were provided by D. W. Litchfield (University of Western Ontario, Ontario, Canada; Ref. 34). The CK2α K68M or CK2α′ K69M mutants contain a single point mutation in the kinase domain of CK2α and CK2α′ catalytic subunits, respectively, and are devoid of kinase activity (34). The pRc/CMV2 plasmid contains a *neo*-resistance gene driven by the CMV promoter (Invitrogen Life Technology). To establish stable transfectants, P100 dishes of cells were transfected with 20 μg of the appropriate pRc-CMV constructs. After 48 h, cells were selected with 600 μg/ml geneticin (Invitrogen Life Technology) for 10 days, and then grown in the presence of 100 μg/ml geneticin. Alternatively, cells were cotransfected with 1 μg of the pGKpuro selection plasmid, selected with 4 μg/ml puromycin (Sigma) for 4 days, and then grown in the presence of 1 μg/ml puromycin.

Retroviral Gene Delivery. The murine CK2α cDNA (22) was excised from the pcDNA3.0 vector (Invitrogen Life Technologies, Inc.) by *Bam*HI digestion and subcloned into the *Bam*HI site of the pBabe-puro ecotropic retroviral vector (35), yielding pBabe-puro-CK2α. Phoenix packaging cells were used for the generation of retrovirus, which were selected with 500 μg/ml hygromycin to increase Gag and Pol viral protein expression. Briefly, P100 dishes of 80% confluent Phoenix cells were transfected with 15 μg of pBabe-puro-CK2α or pBabe-puro along with 5 μg of an Env-expressing vector. After 24 h, the medium was changed. and cells were incubated for another 24 h at 32°C to increase retrovirus half-life. Supernatants containing retrovirus were then harvested, filtered and transferred on NIH 3T3 target cells in the presence of 2 μg/ml polybrene (Sigma). After 24 h, infected cells were washed, selected with complete medium plus 4 μg/ml puromycin for 4 days, and expanded in medium containing 1 μg/ml puromycin. Single cell clones were isolated by limiting dilution. As a positive control, cells were infected with the pBabe-puro-GFP retrovirus, indicating more than 90% efficiency in retroviral infection of NIH 3T3 cells (data not shown).

EMSA. The sequence of the WT URE NF-κB-containing oligonucleotide from the c-*myc* gene is as follows: WT, 5′-AAGTCCGGGTTTTCCCCAACC-3′ (36). The core element is underlined. The mutant URE has a two G-to-C-bp conversion, indicated in bold, which blocks NF-κB/Rel binding: 5′-AAGTCC**GC**CTTTTCCCCAACC-3′. The sequence of the Sp1 oligonucleotide is 5′-ATTCGATCGGGGCGGGGCGACC-3′. The Oct1 oligonucleotide has the following sequence: 5′-TGTCGAATGCAAATCACTAGAA-3′. Nuclear extracts from cell lines were prepared and samples (2.5–5 μg) subjected to EMSA as described (24). For antibody supershift analysis, the binding reaction was performed in the absence of the probe, the appropriate antibody was added, and the mixture incubated for 16 h at 4°C. The probe was then added and the reaction incubated an additional 30 min at 25°C and the complexes resolved by gel electrophoresis, as above. Antibodies used included: anti-RelA (C-20): sc-372, anti-p50 (NLS): sc-114, anti-p52 (K-27): sc-298; and anti-c-Rel (C): sc-71 (all from Santa Cruz Biotechnology, Santa Cruz, CA). When indicated, 250 ng of either IκB-α-GST fusion protein or GST alone or excess unlabelled WT or mutant oligonucleotide was added to the binding reaction just before addition of the probe. Data were quantified by densitometry using a Molecular Dynamics densitometer.

Immunoblotting. To prepare WCEs, cells were washed with PBS, resuspended in cold PD buffer [40 mM Tris (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenylphosphate, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.1% NP40]. Cells were lysed by sonication, and debris was removed by centrifugation. Samples of WCEs or nuclear extracts, prepared as described above, were separated by electrophoresis in polyacrylamide-SDS gels, transferred to polyvinylidine difluoride membrane (Millipore, Bedford, MA) and subjected to immunoblotting. Monoclonal antibodies specific for HA-tag (F7) and β-actin (AC-15) were purchased from Sigma. The rabbit polyclonal antibody specific for the CK2α subunit of human CK2 (residues 70–89) was from Stressgen (Victoria, British Columbia, Canada). Antibodies specific for mouse IκB-α (C-21), sc-371, and human IκB-α (C-15), sc-203, were from Santa Cruz Biotechnology. Antibodies specific for NF-κB subunits were the same as those used for the supershift assays.

CK2 Kinase Assay. Samples (2 μg) of WCEs were incubated with a 1 mM solution of the CK2-specific peptide substrate RRREEETEEE (Sigma-Genosys Inc.) in a CK2 kinase buffer {100 mM Tris (pH 8.0), 20 mM $MgCl_2$, 100 mM KCl, 0.1 μg/ml BSA, 100 μM Na_3VO_4, 5 μCi [γ-^{32}P]GTP} at 30°C for 10 min. The reaction was stopped by adding 25 μl of 100 mM ATP in 0.4 N HCl. Samples were spotted onto a P81 Whatmann filter and washed four times in 150 mM H_3PO_4 to remove unincorporated [γ-^{32}P]GTP; phosphorylated peptides were measured by scintillation counting. The samples were assayed in duplicate, and the background kinase activity in the absence of the peptide substrate was subtracted. For evaluation of phosphorylation of IκB-α, 10 to 20 μg of WCEs were diluted to 10-μl final volume in PD buffer. After the addition of 15 μl of buffer D {100 mM Tris (pH 8.0), 100 mM NaCl, 50 mM KCl, 20 mM $MgCl_2$, 100 μM Na_3VO_4, and 10 μCi [γ-^{32}P]GTP}, reactions were incubated at 30°C for 30 min in the presence of 200 ng GST-wtIκB-α as substrate. Alternatively, GST-3CIκB-α, with three point mutations at S283A, T291A, and T299A, kindly provided by J. Hiscott, was used as substrate (5). The kinase reaction was stopped by the addition of 2× SDS-PAGE sample buffer, and the mixture subjected to SDS-PAGE analysis and visualized by autoradiography. To evaluate the kinase activity of transfected WT or mutant proteins HA-CK2α or HA-CK2α′ in cell extracts, samples (100 μg) of WCEs were precleared with protein G-Sepharose beads (Amersham Pharmacia Biotech AB, Piscataway, NJ) for 1 h at 4°C. The HA-tagged CK2 proteins were collected using 1 μg of the HA-tag F7 antibody. Controls included immunoprecipitations performed with normal polyclonal mouse IgG (Santa Cruz Biotechnology). After 16 h of incubation and extensive washing, immunoprecipitates were subjected to a CK2 kinase assay, as described above.

TNF-α-induced Apoptosis Assay. Cells were plated at 2×10^4/ml in 96-well plates. After 24 h, cells were treated with recombinant human TNF-α

(PeproTech Inc., Rocky Hill, NJ) in the presence of 30 μg/ml cycloheximide (Sigma). After 16 h, cell viability was evaluated by the nonradioactive MTS cell proliferation assay (Promega, Madison, WI).

Focus Formation Assay. Cells were plated at 3×10^3/ml in top plugs consisting of complete medium and 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, ME). Plates were subsequently incubated for 18 days in humidified incubator at 37°C. Cells were stained with 0.5 ml of 0.0005% crystal violet, and colonies were counted using a dissecting microscope.

RESULTS

Expression of Protein Kinase-inactive CK2α or CK2α′ Inhibits CK2 Activity in NF639 MMTV-Her-2/neu Mammary Tumor Cells. Using the NF639 cell line, which was established from a mammary tumor that arose in a MMTV-Her-2/neu transgenic mouse (30), we demonstrated that Her-2/neu activates classical NF-κB via a noncanonical pathway, *i.e.*, involving calpain- rather than IKK-induced proteasome-mediated degradation of IκB-α (28). Given the recent evidence implicating phosphorylation by CK2 in degradation of IκB-α via calpain (9, 10), we compared expression levels of CK2α catalytic subunit in NF639 cells with levels in untransformed NMuMG mouse mammary epithelial cells (Fig. 1*A*). As control for equal loading, samples were similarly analyzed for levels of β-actin (Fig. 1*A*). Immunoblot analysis revealed an increase in the level of CK2α protein in NF639 cells compared with NMuMG cells. Scanning of this and a duplicate blot analysis indicated that NF639 cells display a 2.8 ± 0.3-fold increase in total level of CK2α compared with NMuMG cells.

To inhibit constitutive CK2 activity in NF639 cells, vectors expressing kinase-inactive CK2α or CK2α′ subunits were used. Stably transfected pools of NF639 cells expressing HA-tagged HA-CK2α K68M or HA-CK2α′ K69M mutants were selected. As controls, cells were transfected with the pRc/CMV2 backbone vector, as well as HA-CK2α WT or HA-CK2α′ WT expression vectors. To examine the kinase activity of exogenously expressed CK2α and CK2α′, ectopic HA-tagged CK2 proteins were immunoprecipitated from WCEs with an HA-specific antibody, and CK2 kinase activity assayed as described previously (20), using as substrate either GST-wtIκB-α or GST-3CIκB-α (with S283A, T291A, and T299A point mutations in COOH-terminal residue targets of CK2 phosphorylation) as a negative control (Fig. 1*B*). In cells expressing HA-tagged CK2α WT or CK2α′ WT, HA-specific immunoprecipitations revealed substantial kinase activity. As expected, no kinase activity was detected with the mutant GST-3CIκB-α as a substrate, or with immunoprecipitates performed with normal mouse IgG. When kinase assays were performed on immunoprecipitates from cells transfected with the pRc/CMV2 backbone vector, no detectable kinase activity was observed. Immunoprecipitates from cells expressing HA-tagged CK2α K68M or CK2α′ K69M displayed less than 5% of the kinase activity seen with immunoprecipitates from cells expressing WT CK2 proteins. This small but reproducible kinase activity suggests that the transfected inactive subunits may form holoenzyme complexes with endogenous WT CK2α- or CK2α′-active subunits. To examine the effect of the kinase-inactive variants on total CK2 activity, we performed a kinase assay on WCEs with the CK2-specific peptide substrate RRREEETEEE (Fig. 1*C*). We observed that NF639 cells expressing CK2α K68M or CK2α′ K69M displayed an ~30–40% drop in total CK2 activity. Therefore, expression of CK2α K68M or HA-CK2α′ K69M resulted in a partial inhibition of constitutive CK2 activity in NF639 cells.

Inhibition of CK2 Reduces Basal NF-κB Binding Activity and Stabilizes IκB-α Degradation in Her-2/neu-induced NF639 Breast Cancer Cells. We next measured the effects of CK2 inhibition on the Her-2/neu-induced NF-κB activity in NF639 cells, which we described previously (28). To assess NF-κB binding levels, nuclear extracts were prepared from NF639 cells expressing HA-CK2α

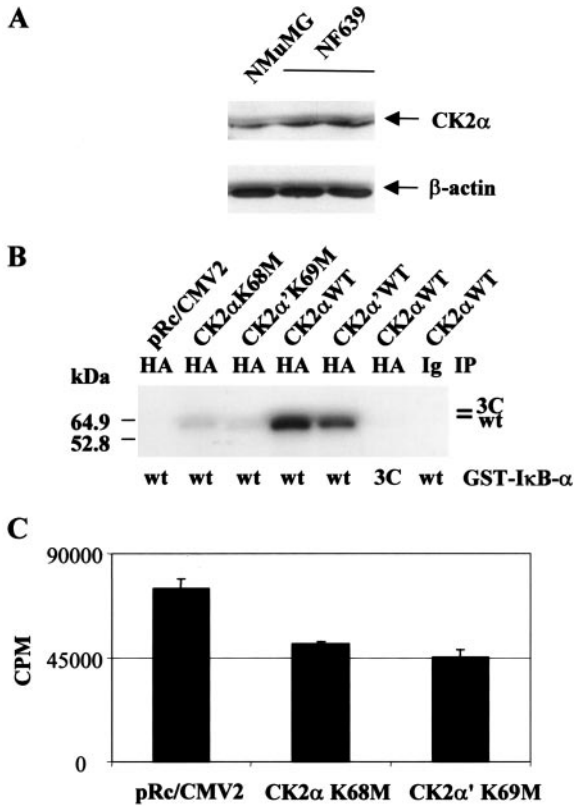

Fig. 1. Expression of kinase-inactive CK2α or CK2α′ subunit inhibits CK2 activity in Her-2/neu-induced NF639 breast cancer cells. *A*, CK2α expression. WCEs were prepared from two independent cultures of NF639 and NMuMG untransformed mouse breast epithelial cells at 70% confluence. Samples (80 μg) were subjected to immunoblot analysis for CK2α and β-actin levels. *B*, expression of transfected CK2 constructs in NF639 cells. Cells were transfected with parental pRc/CMV2, HA-CK2α K68M, HA-CK2α′ K69M, HA-CK2α WT, or HA-CK2α′ WT plasmid expression vectors. Stably transfected cells were selected with puromycin. WCEs were prepared, and equal amounts (300 μg) were immunoprecipitated with either the F7 antibody against HA-tag (HA) or normal mouse IgG (*Ig*). Immunoprecipitates were subjected to a CK2 kinase assay in the presence of [γ-^{32}P]GTP using either GST-wtIκB-α or, as a negative control, GST-3CIκB-α (*3C*, with three point mutations at S283A, T291A, and T299A in the COOH-terminal PEST domain). Proteins were resolved by PAGE-SDS and visualized by autoradiography. Positions of molecular weight protein standards of M_r 64,900 (*64.9*) and 52,800 (*52.8*) and of the GST-wtIκB-α (*wt*) and GST-3CIκB-α (*3C*) proteins are as indicated. *C*, total CK2 activity. NF639 cells were transfected with the indicated constructs and selected with geneticin. Samples of WCEs (2 μg) were assayed in duplicate for CK2 phosphorylation using a specific CK2 substrate peptide. Data are expressed as the means with background (cpm in the absence of peptide) subtracted.

K68M, HA-CK2α′ K69M, or both kinase-inactive variants combined, and were subjected to EMSA. As probe, an oligonucleotide containing the NF-κB element upstream of the c-*myc* promoter was used and Oct1 served as a control for equal loading (Fig. 2*A*). Two major NF-κB complexes were observed with extracts from the NF639 cell line, which have been identified previously as p50/RelA and p50 homodimer complexes (28). Expression of kinase-inactive CK2α or kinase-inactive CK2α′ caused a significant reduction in both p50/RelA and p50/p50 binding levels compared with transfection with the pRc/CMV2 plasmid. Coexpression of CK2α K68M and CK2α′ K69M mutants did not have an additive effect.

To further study the effect of inhibition of CK2 on cellular localization of NF-κB subunits, immunoblot analysis was performed for RelA, p50, and p52 NF-κB subunits in WCEs and nuclear extracts from CK2α K68M-expressing NF639 cells or pRc/CMV2-transfected control cells (Fig. 2*B*). Results showed that nuclear levels of p50 and RelA were significantly lower in CK2α K68M-expressing cells compared with the control cells, whereas total levels were increased in the cells, presumably localized in the cytoplasm. In contrast, no changes

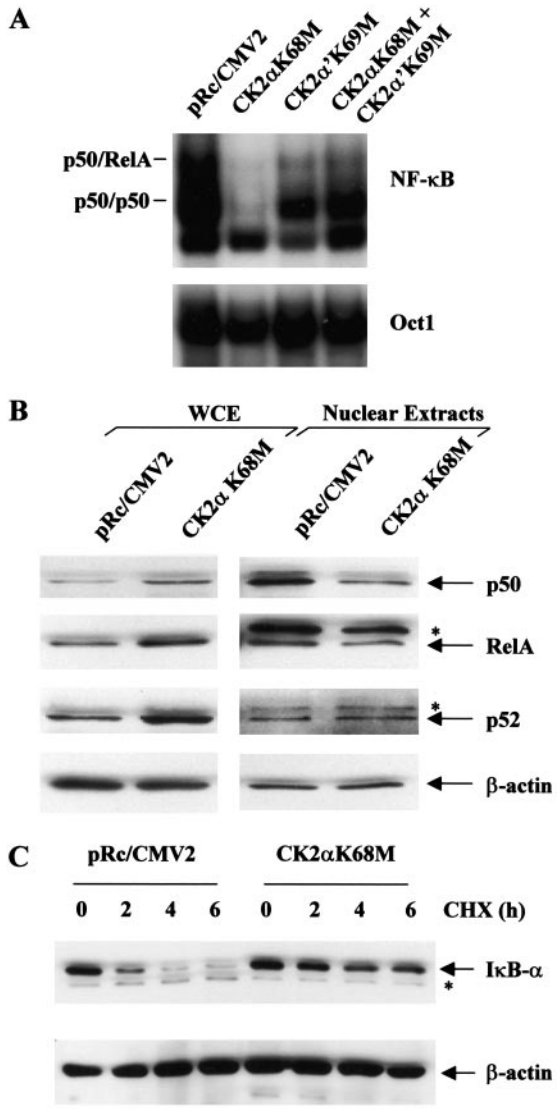

Fig. 2. Expression of kinase-inactive CK2α or CK2α' subunits inhibits constitutive NF-κB binding activity in NF639 breast cancer cells. Cells were transfected and mixed populations of stably transfected cells selected with puromycin. *A*, NF-κB binding activity. Nuclear extracts were prepared and samples (5 μg) subjected to EMSA; probes were oligonucleotides containing either NF-κB- or Oct1-binding elements. The position of the p50/p50 and p50/RelA complexes, identified previously (28), are as indicated. Similar results were observed with two additional independent sets of NF639 cells stably transfected with CK2 mutant constructs (data not shown). *B*, NF-κB subunits: total and nuclear levels. WCEs and nuclear extracts were prepared, and samples (50 μg of WCEs and 5 μg of nuclear extracts) were subjected to SDS-PAGE and immunoblot analysis of p50, RelA, p52, and β-actin, as loading control. *, positions of nonspecific bands. *C*, IκB-α half-life. Exponentially growing cells were incubated with 50 μg/ml cycloheximide (*CHX*), and WCEs were prepared at 0, 2, 4, and 6 h. Samples (50 μg) were subjected to immunoblot analysis for IκB-α, and β-actin, as loading control. *, the position of a nonspecific band.

could be detected in nuclear p52 levels. Equal loading was confirmed by an analysis of β-actin levels. Therefore, the inhibition of CK2 results in reduced NF-κB binding caused by decreased nuclear levels of RelA and p50 proteins.

CK2-mediated phosphorylation of IκB-α in the COOH-terminal PEST domain has been implicated in the basal and signal-dependent turnover of free and NF-κB-bound IκB-α (4–8). We next tested the involvement of CK2-mediated phosphorylation in the rate of IκB-α turnover in NF639 cells. Exponentially growing NF639 cells, stably transfected with HA-CK2α K68M or parental vectors, were treated with the protein synthesis inhibitor cycloheximide and WCEs prepared at the times indicated and subjected to immunoblotting for IκB-α expression (Fig. 2*C*). The stability of IκB-α protein was substantially increased in NF639 cells upon inhibition of CK2. In two independent sets of stably transfected NF639 cells, the half-life of IκB-α decay was between ~2 and 4 h in pRc/CMV2-transfected control cells and increased to more than 6 h in cells expressing CK2α K68M (Fig. 2*C*, and data not shown). Overall, these results demonstrate the ability of protein kinase CK2 to affect NF-κB levels in Her-2/neu-induced NF639 breast cancer cells via the regulation of IκB-α phosphorylation and degradation.

Inhibition of Protein Kinase CK2 Sensitizes Her-2/neu-induced NF639 Mouse Breast Cancer Cells to Apoptosis and Loss of Anchorage-independent Growth. Work from many laboratories, including our own, have highlighted the importance of constitutive NF-κB activity in protecting breast cancer cells from apoptosis (24). We, therefore, assessed the effect of the inhibition of CK2 on TNF-α-induced cell death. NF639 cells, stably transfected with HA-CK2α K68M expression vector, were treated with cycloheximide and stimulated with an increasing dose of TNF-α (25–400 ng/ml). Cell viability was assessed by MTS assay 24 h after stimulation. As a control, NF639 cells, stably expressing the IκB-α 2N3C (S32A, S36A, S283A, T291A, and T299A) super-repressor mutant, which cannot be phosphorylated by IKK or CK2 kinases and is, therefore, resistant to degradation, were similarly assessed. NF639 cells that expressed IκB-α 2N3C displayed a profound decrease in p50/RelA binding and NF-κB transcriptional activity, as expected (data not shown). Minimal cell death was observed in parental pRc/CMV2-transfected NF639 cells, which is consistent with the elevated NF-κB constitutive levels observed in this cell line (Ref. 28; Fig. 3*A*). In contrast, cells expressing CK2α K68M or IκB-α 2N3C displayed increased susceptibility to TNF-α-induced cell death, as judged by MTS assay. Results obtained with this and a second independent set of stably transfected cells were quantified. At a concentration of 400 ng/ml TNF-α, the percentage of viable pRc/CMV2-, CK2α K68M-, or IκB-α 2N3C-expressing NF639 cells was 85.0 ± 5.6%, 52.5 ± 4.9% ($P < 0.001$), and 58.5 ± 12.1% ($P < 0.03$), respectively. Therefore, the inhibition of CK2 activity sensitizes NF639 cells to TNF-α-induced cell death.

We next asked whether the inhibition of CK2 affected the transformed phenotype of NF639 breast cancer cells. Cultures of two independent sets of NF639 cells stably expressing kinase-inactive CK2α were assessed for growth in soft agar (Fig. 3*B*). The numbers of colonies per high power field with the parental pRc/CMV2 *versus* CK2α K68M vectors were as follows: 124 ± 20 and 37 ± 18 ($P < 0.01$, first set of cell populations), and 107 ± 13 and 38 ± 6 ($P < 0.002$, second set of cell populations). Thus, the inhibition of CK2 activity leads to a substantial reduction in the transformed phenotype of Her2/neu breast cancer cells, as measured by the loss of anchorage-independent growth of these cells.

Expression of Kinase-inactive CK2α Inhibits NF-κB Activity in Hs578T Human Breast Cancer Cells and 293T Human Embryonic Kidney Cells. We next asked whether the inhibition of CK2 activity in other cell lines would similarly decrease NF-κB activity. The Hs578T human breast tumor cell line, which overexpresses CK2 protein and kinase activity (20), was selected for study. Hs578T cells were identified previously to express predominantly activated p50/RelA and p50/p50 NF-κB complexes (20, 24). For these experiments, mixed cell populations of Hs578T cells stably expressing HA-tagged mutant CK2α subunit were obtained by geneticin selection. Nuclear extracts and WCE were prepared and analyzed for NF-κB binding levels and expression of HA-tagged CK2, respectively (Fig. 4*A*). Results obtained with this and a second set of stably transfected cells showed an ~50% drop in NF-κB binding in HA-CK2α K68M-expressing Hs578T cells compared with parental pRc/CMV2-transfected cells. Expression of CK2α K68M mutant was confirmed by

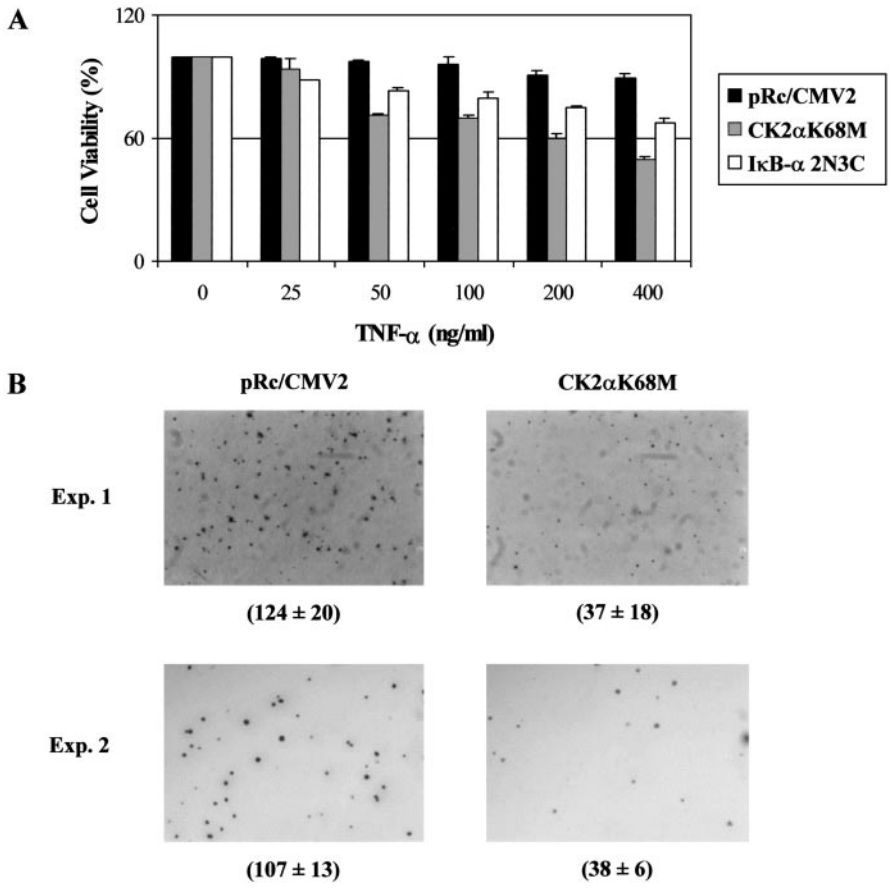

Fig. 3. Expression of kinase-inactive CK2α subunit increases susceptibility to TNF-α-induced apoptosis and reduces anchorage-independent growth of NF639 breast cancer cells. *A*, TNF-α-induced cell death. Mixed population of NF639 cells stably transfected with HA-CK2α K68M, parental pRc/CMV2, or IκB-α 2N3C super-repressor plasmid expression vectors were plated at 2×10^3 in 96-well plates. After 24 h, cells were treated with recombinant human TNF-α in the presence of 30 μg/ml cycloheximide. After 16 h, cell viability was evaluated by the MTS cell proliferation assay. Results are expressed as percentage of viable cells (absorbance at 490 nm normalized to absorbance of cultures treated with cycloheximide alone). *B*, soft agar assay. Cells were plated, in triplicate, at 3×10^3/ml in top plugs consisting of complete medium and 0.4% agarose. After 18 days, the numbers of foci were scored and pictures taken with a Nikon camera ($\times$1.5). Results were obtained with two independent sets of transfected cells. *Below the images*, values of colony number per high power field.

immunoblotting for the HA tag. Thus, the inhibition of CK2 similarly reduces NF-κB binding in Hs578T human breast cancer cells.

To assess whether the regulation of CK2 activity could be extended to another cell type, 293T human embryonic kidney cells were similarly tested. Because transfection of these cells occurs with a high efficiency, transient transfections were performed with either HA-CK2α K68M or HA-CK2α' K69M expression vectors or the parental vector, as control. Cells were harvested 6 days after transfection to ensure substantial expression of ectopic proteins compared with endogenous proteins. As seen in Fig. 4*B*, expression of kinase-inactive CK2α or CK2α' mutants resulted in significant decreases in NF-κB binding. An 89% and 76% drop in band 1 and band 2, respectively, was noted in 293T cells expressing CK2α' K69M compared with parental cells. In 293T cells, the CK2α' K69M was found to have a stronger effect than CK2α K68M, consistent with the higher expression level of transiently expressed CK2α' mutant compared with CK2α mutant that was routinely seen (Fig. 4*B*, bottom panel, and data not shown). These results extend the finding on the ability of kinase-inactive forms of CK2 to reduce constitutive NF-κB levels to 293T human embryonic kidney cells.

CK2α Overexpression in NIH 3T3 Cells by Retroviral Gene Delivery Leads to Increased Nuclear NF-κB Expression. To determine whether increased CK2 expression is sufficient to induce NF-κB levels, we next attempted to increase CK2 activity through overexpression of the CK2α catalytic subunit of CK2. We turned to the NIH 3T3 fibroblast cell line, which has lower levels of endogenous CK2 than the NF639 or Hs578T cell lines (data not shown). Cells were retrovirally infected with a vector expressing an untagged murine CK2α, and a mixed population of infected cells as well as single clones were selected in puromycin and were screened for total CK2α protein levels. Two stable CK2α-overexpressing NIH 3T3 clones, designated Clone 4 and Clone 6, were chosen for this study. To monitor the relative levels of CK2α expression in the selected cells, immunoblot analysis was performed with a CK2α polyclonal antibody using samples of WCEs and nuclear protein extracts (Fig. 5*A*). Two bands were detected in the WCEs. The top band corresponded to the full length CK2α (M_r ~45,000), and the bottom one to a protein of M_r ~40,000. Immunoblot analysis performed with antibodies specific for either the NH_2-terminal or COOH-terminal part of CK2α indicated that the M_r ~40,000 protein likely resulted from COOH-terminal clipping of CK2α (data not shown), as described previously during the *in vitro* purification of human recombinant CK2α (12). The WCEs of the Babe-CK2α mixed population, Clone 4, and Clone 6 demonstrated elevated levels of CK2α protein compared with the parental vector control (Babe)-infected NIH 3T3 cells, which showed only low basal expression. When the blots were scanned and normalized to β-actin levels, a 2.6-, 15.1-, and 9.5-fold increase in CK2α expression was observed in NIH 3T3 Babe-CK2α mixed-population cells, Clone 4, and Clone 6, respectively, compared with the parental Babe cells. No significant increase in CK2α' was observed in Babe-CK2α cells (data not shown). Higher levels of CK2α expression were also detected in the nuclei of Babe-CK2α cells (Fig. 5*A*). These results were confirmed by immunofluorescent staining of CK2α, which showed a strong accumulation of CK2α in both the cytoplasm and the nuclei of Clone 4 and Clone 6 compared with parental NIH 3T3 Babe cells (data not shown). To confirm that increased CK2α expression led to increased CK2 enzymatic activity, a CK2 kinase assay was performed with GST-wtIκB-α or GST-3CIκB-α as substrates. WCEs, prepared from NIH 3T3 Babe-CK2α mixed population and clones, were used directly in *in vitro* CK2 phosphorylation assays (Fig. 5*B*). Kinase assays demonstrated strong preferential phosphorylation of GST-wtIκB-α compared with GST-3CIκB-α, consistent with the assay measuring CK2 activity. The mixed population, Clone 4, and Clone 6 displayed an increase in CK2 kinase

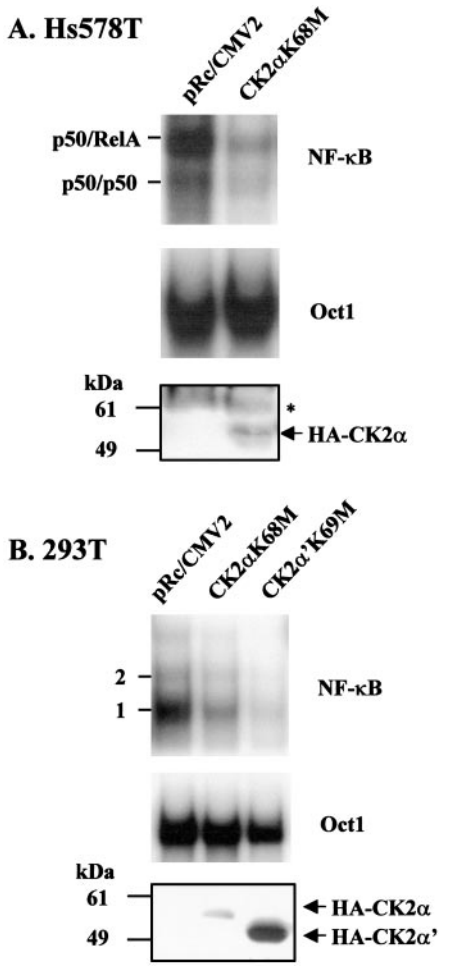

Fig. 4. Expression of kinase-inactive CK2α or CK2α′ subunit inhibits NF-κB binding activity in Hs578T human breast cancer cells and 293T human embryonic kidney cells. *A*, Hs578T cells. Mixed population of cells, stably transfected with pRc/CMV2 or HA-CK2α K68M plasmid vectors, were selected in geneticin, and nuclear extracts and WCEs were prepared. *Top panel*, samples of nuclear extracts (5 μg) were subjected to EMSA for NF-κB or Oct1. The position of the p50/p50 and p50/RelA complexes identified previously (20) are as indicated. *Bottom panel*, samples of WCE (80 μg) were subjected to immunoblot analysis of exogenous CK2α, using an HA tag-specific antibody. *, the position of a nonspecific band. The position of molecular weight protein standards of M_r 61,000 (*61*) and M_r 49,000 (*49*) are as indicated. *B*, 293T cells. Cells were transiently transfected with parental pRc/CMV2, HA-CK2α K68M, or HA-CK2α′ K69M plasmid vectors. Cells were harvested after 6 days and analyzed as described in *A*. *Band 1* and *band 2*, the two major NF-κB complexes.

activity of 1.2-, 2.3-, and 1.6-fold, respectively, compared with the parental NIH 3T3 Babe cells. Although the magnitude of increase in activity is less than the increase in protein, these results confirm that the two clones and the mixed population of cells display elevated CK2 activity.

We next assessed NF-κB binding levels by EMSA (Fig. 6). Nuclear extracts from the parental NIH 3T3 Babe cells displayed low levels of two NF-κB binding complexes, as observed previously (37). NIH 3T3 BabeCK2α mixed population of cells displayed increased levels of the two NF-κB complexes (Fig. 6*A*). Higher levels of these complexes were detected in both the Clone 4 and Clone 6 cells. When these results were scanned, a 1.5- and 2.5-fold increase in band 1 and band 2, respectively was noted in Clone 4 cells, and a 1.7- and 2.9-fold increase in band 1 and band 2, respectively, was noted in Clone 6 cells, compared with parental Babe cells. To determine the composition of NF-κB complexes, antibody supershift analysis was performed using extracts from Clone 4 cells (Fig. 6*B*). The major NF-κB complexes appeared to consist of p50/RelA heterodimers (Fig. 6*B*, *band 2*) and homodimers of p50 (Fig. 6*B*, *band 1*). No binding of other NF-κB subunits such as p52 or c-Rel was seen. Similar results were obtained with the parental Babe cells (data not shown). Successful competition with WT but not mutant oligonucleotide and inhibition upon addition

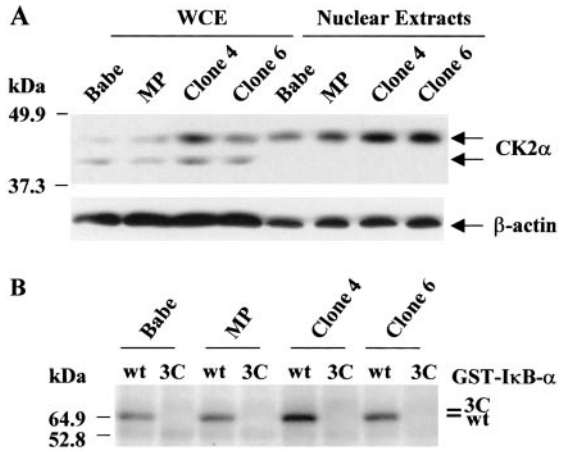

Fig. 5. Overexpression of the CK2α subunit in NIH 3T3 cells. NIH 3T3 cells were infected with recombinant retrovirus for pBabe (*Babe*) or pBabe-CK2α vectors. A mixed population of Babe-CK2α (*MP*) cells and two clones (*Clone 4* and *Clone 6*) were selected in puromycin. *A*, CK2α expression. Samples of WCEs (80 μg) or nuclear extracts (15 μg) from the indicated cells were separated by SDS-PAGE and subjected to immunoblot analysis for CK2α and β-actin levels. Two bands are seen in the WCEs with the CK2α antibody. *Top band*, the full-length CK2α protein; *bottom band*, (likely) a COOH-terminal clipped form of CK2α. *B*, CK2 activity. WCEs were prepared from the indicated cell populations, and samples (10 μg) were subjected to a CK2 kinase assay using as substrate either GST-wtIκB-α (*wt*) or GST-3CIκB-α (*3C*), as a negative control.

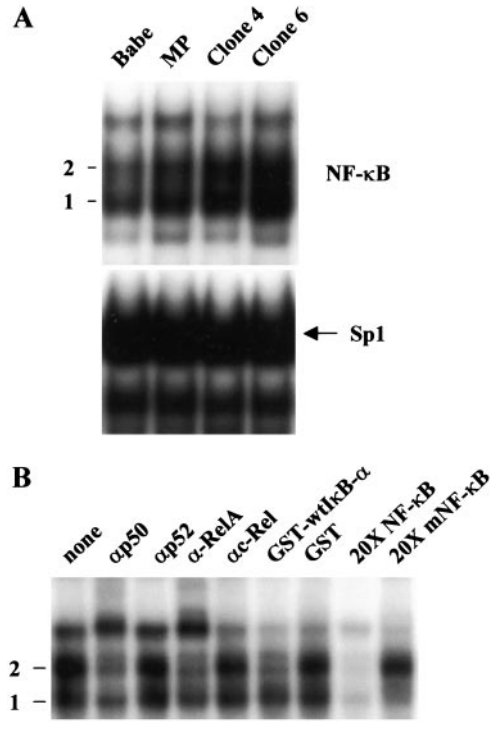

Fig. 6. Overexpression of CK2α subunit results in increased NF-κB binding in NIH 3T3 cells. *A*, NF-κB binding activity. Nuclear extracts were prepared from the indicated cells, and samples (5 μg) were subjected to EMSA with oligonucleotides specific for NF-κB and Sp1. *Band 1* and *band 2*, the positions of the two major NF-κB complexes. *B*, supershift analysis. Nuclear extracts from NIH 3T3 Babe-CK2α Clone 4 cells were incubated in the absence (*none*), or presence of antibody (α) against p50, p52, RelA, or c-Rel NF-κB subunits, or 1 μg GST-wtIκB-α or GST protein, and were subjected to EMSA with a NF-κB oligonucleotide. To test for binding specificity, the binding reaction was performed in the presence of 20× excess unlabelled WT (*20X NF-κB*) or mutant (*20X mNF-κB*) NF-κB oligonucleotide.

of GST-wtIκB-α confirmed the specificity of the NF-κB binding (Fig. 6*B*). Thus, overexpression of CK2α in NIH 3T3 cells leads to up-regulation of classical NF-κB binding.

CK2α Overexpression in NIH 3T3 Cells Leads to Increased Turnover of IκB-α and NF-κB Transcriptional Activity. To determine whether the increase in NF-κB binding upon overexpression of CK2α is caused by increased IκB-α degradation, exponentially growing NIH 3T3 Babe-CK2α Clone 4, Clone 6 and parental Babe cell lines were incubated in the presence of cycloheximide for a period of 0, 4, 8, or 12 h. WCEs were isolated and subjected to immunoblotting for IκB-α expression (Fig. 7*A*, *left panel*). The results were scanned, and the half-life of decay of IκB-α protein was >12 h in parental Babe cells, whereas it was 8.1 and 5.9 h in Babe-CK2α Clone 4 and Clone 6 cells, respectively (Fig. 7*A*, *right panel*). In this and two duplicate experiments, IκB-α protein decayed with a $t_{1/2}$ of 10.2 ± 0.3 h, 5.4 ± 1.4 h ($P < 0.01$), and 6.0 ± 0.3 h ($P < 0.001$) in parental Babe, Clone 4, and Clone 6 cells, respectively. Thus, IκB-α is more rapidly degraded in the CK2-overexpressing cells. To confirm the involvement of CK2 in enhanced IκB-α degradation, Babe-CK2α clones were transfected with human IκB-α WT, 2N (S32A, S36A) mutant or 3C (S283A, T291A, and T299A) mutant in plasmid vectors. The half-lives of IκB-α WT and 2N proteins were found to be quite similar, and much shorter than that of IκB-α 3C mutant, which cannot be phosphorylated by CK2 (data not shown), consistent with the rate of decay of IκB-α protein dependent on CK2-phosphorylation.

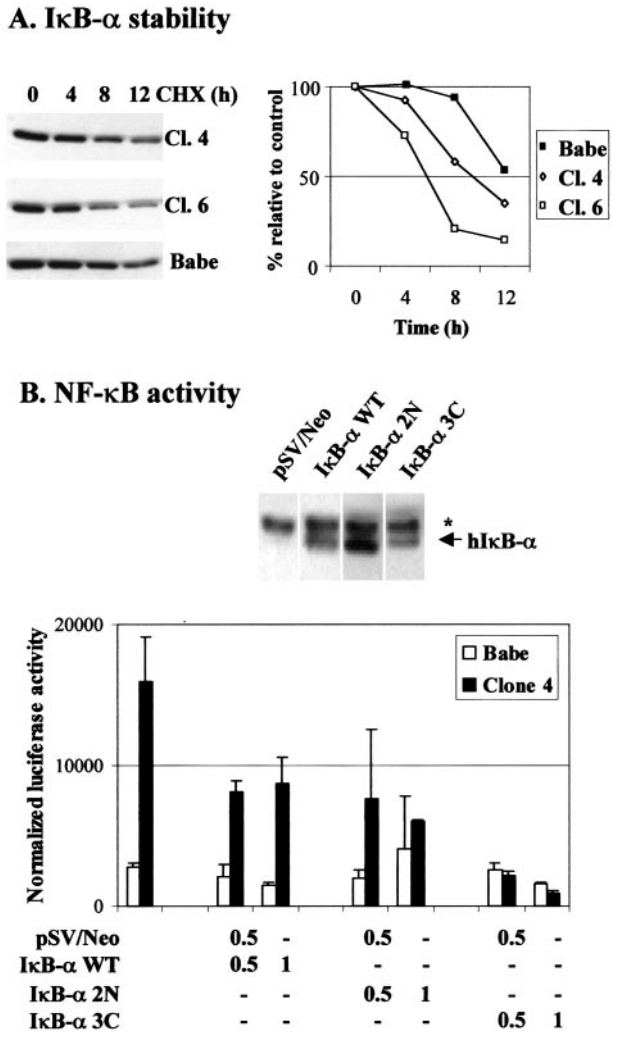

Fig. 7. Overexpression of CK2α subunit results in a higher rate of IκB-α degradation and NF-κB transcriptional activity in NIH 3T3 cells. *A*, IκB-α half-life. *Left panel*, the indicated cells, in exponential growth, were treated with 50 μg/ml cycloheximide (*CHX*), and WCEs were prepared at 0, 4, 8, or 12 h. Samples (50 μg) were subjected to immunoblot analysis for IκB-α. *Right panel*, the immunoblots in the left panel were subjected to densitometry, and the results were presented as the percentage relative to the control. *B*, NF-κB transcriptional activity. *Lower panel*, cultures of parental Babe or Clone 4 cells were transiently transfected, in triplicate, with 1 μg of NF-κB element-driven luciferase reporter construct and 0.5 μg of pSV40-β-gal in the presence of 0.5 or 1 μg of pSV/Neo empty parental vector or of the human IκB-α construct vectors: WT (*WT*), 2N mutant (S32A, S36A), or 3C mutant (S283A, T291A, and T299A in the COOH-terminal PEST domain). After 48 h, cultures were harvested, normalized for β-gal activity, and assayed for luciferase activity. Values in the bar graph, luciferase normalized for β-gal activity. *Top panel*, Babe-CK2α Clone 4 cells were transfected with equal amounts of pSV/Neo empty parental vector or human IκB-α construct vectors: WT, 2N mutant, or 3C mutant. After 18 h, WCEs were prepared, and samples (50 μg) were subjected to immunoblot analysis for human IκB-α (*hIκB-α*) using a human-specific antibody. *, the position of a nonspecific band. (All lanes are from the same gel.)

We next assessed the effect of overexpression of CK2α on NF-κB transcriptional activity, comparing parental Babe and Babe-CK2α Clone 4 cells. The cells were transfected with vectors expressing a NF-κB element luciferase reporter plus pSV40-β-gal, for normalization (Fig. 7*B*). Clone 4 cells displayed an ~5.5-fold increase in NF-κB transcriptional activity compared with parental cells. To confirm that CK2-mediated activation of NF-κB transcriptional activity is dependent on COOH-terminal PEST phosphorylation and degradation of IκB-α, cells were transfected with 0.5 or 1 μg of the IκB-α 3C mutant (S283A, T291A, and T299A), the IκB-α WT, or the IκB-α 2N mutant (S32A, S36A). The IκB-α 3C was much more effective at inhibiting NF-κB transcription in Clone 4 cells than were the WT IκB-α or the IκB-α 2N mutant, which cannot be phosphorylated by the IKKs. At the higher dose of plasmid transfection, expression of the IκB-α 3C mutant resulted in a 95% decline in NF-κB activity, whereas expression of IκB-α WT or IκB-α 2N mutant caused a drop of 46 and 62%, respectively, in NF-κB transcriptional activity. Western blot analysis indicated that levels of IκB-α 3C were lower or comparable with those of IκB-α WT or IκB-α 2N after transient transfection in Clone 4 cells (Fig. 7*B*). Thus, these results confirm that phosphorylation of IκB-α in the COOH-terminal PEST domain is physiologically relevant in regulating NF-κB activity in these cells.

DISCUSSION

Here we show that the inhibition of elevated CK2 activity in cancer cells reduces constitutive NF-κB activity, whereas ectopic expression of CK2 is sufficient to induce NF-κB activity in NIH 3T3 fibroblasts. Thus, CK2 plays a pivotal role in the regulation of constitutive NF-κB activity. Importantly, we show for the first time that the inhibition of CK2 activity decreases Her-2/neu-induced NF-κB activity. Thus, kinase-inactive CK2α subunit decreased nuclear NF-κB and transformed phenotype, whereas it increased sensitivity to TNF-α induced death in MMTV-Her-2/neu-derived NF639 mouse mammary carcinoma cells. Similarly, the inhibition of CK2 in Hs578T cells, which also display elevated basal CK2 activity (20), decreased NF-κB binding. Conversely, CK2α overexpression in NIH 3T3 cells was sufficient to increase IκB-α turnover and basal NF-κB activity. Previously, we and others demonstrated that primary breast cancer samples from patients or from a carcinogen-induced rodent model as well as breast cancer cell lines display increased CK2 activity (18–21) and aberrant activation of NF-κB (23, 24, 26, 27), whereas only low levels of CK2 and NF-κB activation were detected in normal breast epithelial cells. Our findings here demonstrate a direct link between overexpression of CK2 and NF-κB in these cancers. Furthermore, they suggest that CK2 kinase is a downstream mediator of Her-2/neu signaling and, thus, represents a potential new therapeutic target for the treatment of these malignancies.

In our studies, we made use of CK2α K68M or CK2α′ K69M mutants, which display a single point mutation in the kinase domain

of these catalytic subunits and which are devoid of kinase activity (34). In the breast cancer cells, these inactive subunits were capable of reducing CK2 activity, *i.e.*, acting as dominant negatives, although they do not act this way in all cells (Ref. 16; as discussed below). In particular, we observed a 30–40% inhibition of total CK2 activity in NF639 cells expressing CK2α K68M or CK2α' K69M as compared with parental cells. The inhibition of CK2 resulted in a drop in NF-κB binding in both NF639 and Hs578T breast tumor cells as well as in 293T human embryonic kidney cells. Thus, CK2 inhibition had a direct affect on NF-κB activity in various cell types. Consistent with these observations, we observed previously that treatment with the selective pharmacological inhibitors of CK2, apigenin or emodin, inhibited NF-κB activity in human breast cancer cell lines (20), and in mouse B-cell lymphomas (10). Interestingly, whereas ectopic expression of WT mouse CK2α catalytic subunit in NIH 3T3 fibroblast cells, which display low basal CK2 activity, led to a substantial increase in CK2 activity and NF-κB binding and activity, we were unable to similarly increase levels of CK2 protein or activity in NF639 cells (data not shown). Interestingly, the NF-κB activity in CK2α-overexpressing NIH 3T3 cells consisted predominantly of classical p50 and RelA-containing complexes, similar to breast tumor tissue from transgenic mice overexpressing CK2α in the mammary gland (19).

We observed previously that Her-2/neu activates NF-κB via a PI3-K-to-Akt-kinase signaling pathway that can be inhibited via antibody against the receptor or by the tumor suppressor PTEN in NF639 breast cancer cells (Ref. 28; see Fig. 8). Different mechanisms may be involved in the regulation of NF-κB activity by CK2. Previous work has indicated that basal and signal-dependent turnover of free and NF-κB-bound IκB-α is controlled by phosphorylation of residues in the COOH-terminal PEST domain by CK2, and, thus, mutation of these sites results in longer half-life of the IκB protein (5, 7, 8). Our evidence indicates that the level of CK2 activity affects IκB-α stability in NF639 cells (Fig. 8). Previously, we demonstrated that the dominant proteolytic pathway for IκB-α degradation in NF639 cells is mediated via calpain (28); and, because in B cells, we observed that CK2 phosphorylation accelerates degradation of IκB by calpain (10), it is possible that a similar mechanism occurs in breast cancer cells. The mechanisms leading to enhanced CK2 activity and the potential role of any additional kinases in the signaling pathway remain to be determined (Fig. 8). In addition, CK2 has been proposed to control NF-κB transcriptional activity by direct phosphorylation of the RelA subunit in response to TNF-α stimulation (38, 39). Here, we observed that NF-κB in CK2α-overexpressing NIH 3T3 cells is clearly nuclear and transcriptionally active in a reporter assay; however, it remains to be determined whether CK2 has a similar affect on basal activity of RelA in breast cancer cells. In addition, CK2 phosphorylates PTEN directly (40–42). Phospho-PTEN has increased stability but reduced lipid phosphatase activity (40), thereby promoting Akt phosphorylation and activation (42). However, no significant change in the activity of Her-2/neu receptor or in the levels of phosphorylated Akt was evident in NF639 breast cancer cells transduced with the kinase-inactive CK2 subunits (data not shown), which suggests that either PTEN expression itself is down-regulated or PTEN phosphorylation cannot be reduced by the kinase-inactive CK2 constructs.

CK2 has been reported to affect cell growth and transformation. Dysregulated expression of both CK2α and CK2α' affect cell proliferation, transformation, and survival; although, the effects of ectopic CK2 subunit expression appear to depend on the type of cells used. For instance, inducible transient expression of kinase-inactive CK2α' K69M in the presence of ectopic CK2β led to a strong attenuation of proliferation in the human osteosarcoma U2-OS cell line, although it did not inhibit total CK2 activity (16). Li *et al.* (15) reported that expression of ectopic Myc-tagged CK2α increased total CK2 activity and moderately

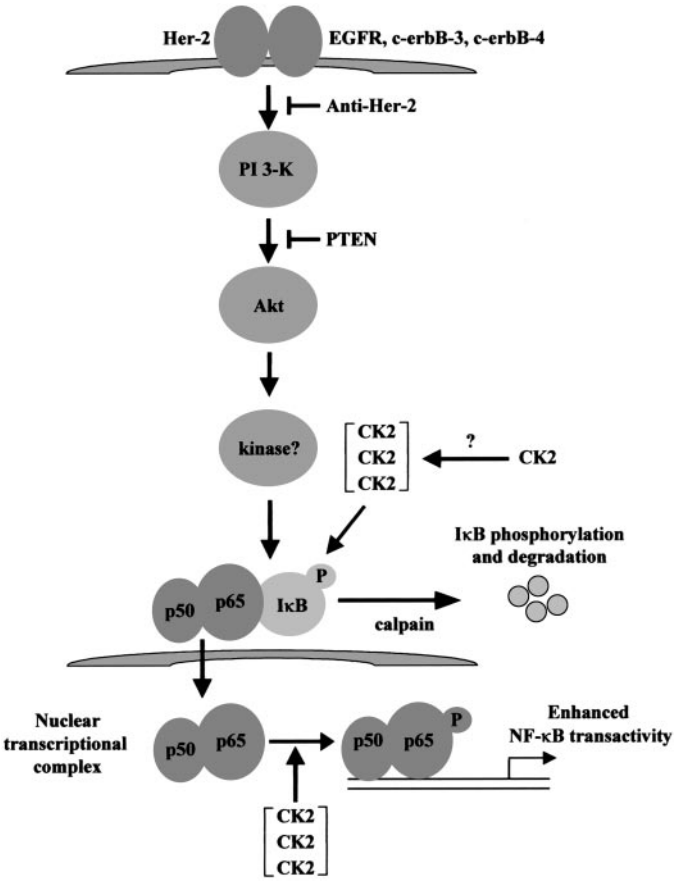

Fig. 8. Scheme of CK2 involvement in Her-2/neu-mediated NF-κB activation in breast cancer cells. Overexpression of Her-2/neu and heterodimerization with other EGF receptor family members (*EGFR, c-erbB-3, c-erbB-4*) activate NF-κB via a PI3-K-to-Akt-kinase signaling pathway that can be inhibited via treatment with an anti-Her-2/neu antibody or the tumor suppressor PTEN (28). Increased cytoplasmic and nuclear CK2 expression and activity have been observed in NF639 cells and many human breast cancers, although the mechanisms controlling this increase remain to be elucidated. Overexpression of CK2 leads to increased IκB-α phosphorylation and rate of degradation, which we have shown to be mediated via a calpain proteolytic pathway (28). The possibility that elevated levels of CK2 promotes NF-κB transcriptional activity by direct phosphorylation of its RelA subunit, as seen previously on TNF-α treatment (38, 39), is under investigation in breast cancer cells.

enhanced the growth of Chinese hamster ovary cells, whereas there was only a low level of expression of the ectopic protein, and no change in growth was seen in 3T3 L1 cells. Expression of kinase-inactive CK2α K68A was also reported to impair cell proliferation in both NIH 3T3 and CCL39 fibroblastic cells, and was linked to a defect in G_1-S phase progression (17). The inhibition of CK2α by antisense oligodeoxynucleotides induced apoptosis in human squamous cell carcinoma of the head and the neck (43). In contrast, overexpression of active forms of CK2α or CK2α' had little or no detectable effect on the proliferation of these cells (16, 17); although, they did cooperate with Ha-*ras* in the transformation of rat embryo fibroblasts or BALB/c 3T3 fibroblasts (14). We observed that stable transfection of NF639 breast cancer cells with CK2α K68M only marginally affected cell proliferation, similar to the effect of stable expression of IκB-α super-repressor mutant (data not shown). Of note, expression of CK2α K68M significantly inhibited colony formation in soft agar. Furthermore, CK2α K68M-expressing cells displayed increased susceptibility to TNF-α-mediated cell death, which is tightly controlled by NF-κB. Together, these results suggest a key role of CK2 in the control of transformed phenotype and cell survival.

Aberrant nuclear NF-κB activity has been reported in many cancers (44). Products of several oncogenes such as *Her-2/neu* (28, 45), the EGF receptor signaling pathway (27, 46), and the oncogenic Raf and

Ras proteins (47, 48) induce NF-κB activity in various cell types. CK2 is markedly elevated in a number of hematopoietic and solid tumors (49) and, given our findings that demonstrate the ability of CK2 to directly affect NF-κB activation, it would be interesting to determine whether NF-κB and CK2 can cooperate to induce transformation. Experiments are in progress with bitransgenic mice that overexpress the c-Rel NF-κB family member and CK2α subunit in mammary epithelial cells. Lastly, our findings suggest that combinations of proteasome and calpain inhibitors or IKK and CK2 kinase inhibitors could be more effective than the use of single inhibitors in blocking IκB degradation and NF-κB activation, in promoting tumor cell apoptosis, and in sensitizing cancer cells to the proapoptotic effects of radiation or chemotherapy.

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REFERENCES

1. Ghosh, S., and Karin, M. Missing pieces in the NF-κB puzzle. Cell, *109:* S81–96, 2002.
2. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature (Lond.), *388:* 548–554, 1997.
3. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science (Wash. DC), *278:* 860–866, 1997.
4. Barroga, C. F., Stevenson, J. K., Schwarz, E. M., and Verma, I. M. Constitutive phosphorylation of IκBα by casein kinase II. Proc. Natl. Acad. Sci. USA, *92:* 7637–7641, 1995.
5. Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. Phosphorylation of IκBα in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. Mol. Cell. Biol., *16:* 1401–1409, 1996.
6. McElhinny, J. A., Trushin, S. A., Bren, G. D., Chester, N., and Paya, C. V. Casein kinase II phosphorylates IκBα at S-283. S-289, S-293, and T-291 and is required for its degradation. Mol. Cell. Biol., *16:* 899–906, 1996.
7. Schwarz, E. M., Van Antwerp, D., and Verma, I. M. Constitutive phosphorylation of IκBα by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IκBα. Mol. Cell. Biol., *16:* 3554–3559, 1996.
8. Pando, M. P., and Verma, I. M. Signal-dependent and -independent degradation of free and NF-κB-bound IκBα. J. Biol. Chem., *275:* 21278–21286, 2000.
9. Miyamoto, S., Seufzer, B. J., and Shumway, S. D. Novel IκBα proteolytic pathway in WEHI231 immature B cells. Mol. Cell. Biol., *18:* 19–29, 1998.
10. Shen, J., Channavajhala, P., Seldin, D. C., and Sonenshein, G. E. Phosphorylation by the protein kinase CK2 promotes calpain-mediated degradation of IκBα. J. Immunol., *167:* 4919–4925, 2001.
11. Xu, X., Toselli, P. A., Russell, L. D., and Seldin, D. C. Globozoospermia in mice lacking the casein kinase IIα′ catalytic subunit. Nat. Genet., *23:* 118–121, 1999.
12. Niefind, K., Guerra, B., Ermakowa, I., and Issinger, O. G. Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme. EMBO J., *20:* 5320–5331, 2001.
13. Pinna, L. A., and Meggio, F. Protein kinase CK2 ("casein kinase-2") and its implication in cell division and proliferation. Prog. Cell Cycle Res., *3:* 77–97, 1997.
14. Orlandini, M., Semplici, F., Ferruzzi, R., Meggio, F., Pinna, L. A., and Oliviero, S. Protein kinase CK2α′ is induced by serum as a delayed early gene and cooperates with Ha-ras in fibroblast transformation. J. Biol. Chem., *273:* 21291–21297, 1998.
15. Li, D., Dobrowolska, G., Aicher, L. D., Chen, M., Wright, J. H., Drueckes, P., Dunphy, E. L., Munar, E. S., and Krebs, E. G. Expression of the casein kinase 2 subunits in Chinese hamster ovary and 3T3 L1 cells provides information on the role of the enzyme in cell proliferation and the cell cycle. J. Biol. Chem., *274:* 32988–32996, 1999.
16. Vilk, G., Saulnier, R. B., St Pierre, R., and Litchfield, D. W. Inducible expression of protein kinase CK2 in mammalian cells. Evidence for functional specialization of CK2 isoforms. J. Biol. Chem., *274:* 14406–14414, 1999.
17. Lebrin, F., Chambaz, E. M., and Bianchini, L. A role for protein kinase CK2 in cell proliferation: evidence using a kinase-inactive mutant of CK2 catalytic subunit α. Oncogene, *20:* 2010–2022, 2001.
18. Munstermann, U., Fritz, G., Seitz, G., Lu, Y. P., Schneider, H. R., and Issinger, O. G. Casein kinase II is elevated in solid human tumours and rapidly proliferating non-neoplastic tissue. Eur. J. Biochem., *189:* 251–257, 1990.
19. Landesman-Bollag, E., Romieu-Mourez, R., Song, D. H., Sonenshein, G. E., Cardiff, R. D., and Seldin, D. C. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene, *20:* 3247–3257, 2001.
20. Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D. C., Traish, A. M., Mercurio, F., and Sonenshein, G. E. Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-κB in breast cancer. Cancer Res., *61:* 3810–3818, 2001.
21. Landesman-Bollag, E., Song, D. H., Romieu-Mourez, R., Sussman, D. J., Cardiff, R. D., Sonenshein, G. E., and Seldin, D. C. Protein kinase CK2: signaling and tumorigenesis in the mammary gland. Mol. Cell. Biochem., *227:* 153–165, 2001.
22. Seldin, D. C., and Leder, P. *Casein kinase IIα* transgene-induced murine lymphoma: relation to theileriosis in cattle. Science (Wash. DC), *267:* 894–897, 1995.
23. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol., *17:* 3629–3639, 1997.
24. Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Investig., *100:* 2952–2960, 1997.
25. Kim, D. W., Sovak, M. A., Zanieski, G., Nonet, G., Romieu-Mourez, R., Lau, A. W., Hafer, L. J., Yaswen, P., Stampfer, M., Rogers, A. E., Russo, J., and Sonenshein, G. E. Activation of NF-κB/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis (Lond.), *21:* 871–879, 2000.
26. Cogswell, P. C., Guttridge, D. C., Funkhouser, W. K., and Baldwin, A. S., Jr. Selective activation of NF-κB subunits in human breast cancer: potential roles for NF-κB2/p52 and for Bcl-3. Oncogene, *19:* 1123–1131, 2000.
27. Biswas, D. K., Cruz, A. P., Gansberger, E., and Pardee, A. B. Epidermal growth factor-induced nuclear factor κB activation: a major pathway of cell-cycle progression in estrogen-receptor negative breast cancer cells. Proc. Natl. Acad. Sci. USA, *97:* 8542–8547, 2000.
28. Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R. J., and Sonenshein, G. E. Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IκB-α that can be inhibited by the tumor suppressor PTEN. Oncogene, *20:* 1287–1299, 2001.
29. Hortobagyi, G. N., Hung, M. C., and Buzdar, A. U. Recent developments in breast cancer therapy. Semin. Oncol., *26:* 11–20, 1999.
30. Elson, A., and Leder, P. Protein-tyrosine phosphatase epsilon. An isoform specifically expressed in mouse mammary tumors initiated by v-Ha-ras or neu. J. Biol. Chem., *270:* 26116–26122, 1995.
31. Song, D. H., Sussman, D. J., and Seldin, D. C. Endogenous protein kinase CK2 participates in Wnt signaling in mammary epithelial cells. J. Biol. Chem., *275:* 23790–23797, 2000.
32. Kimmelman, A., Tolkacheva, T., Lorenzi, M. V., Osada, M., and Chan, A. M. Identification and characterization of R-*ras3*: a novel member of the *RAS* gene family with a non-ubiquitous pattern of tissue distribution. Oncogene, *15:* 2675–2685, 1997.
33. Arsura, M., FitzGerald, M. J., Fausto, N., and Sonenshein, G. E. Nuclear factor-κB/Rel blocks transforming growth factor beta1-induced apoptosis of murine hepatocyte cell lines. Cell Growth Differ., *8:* 1049–1059, 1997.
34. Penner, C. G., Wang, Z., and Litchfield, D. W. Expression and localization of epitope-tagged protein kinase CK2. J. Cell Biochem., *64:* 525–537, 1997.
35. Morgenstern, J. P., and Land, H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res., *18:* 3587–3596, 1990.
36. Duyao, M. P., Buckler, A. J., and Sonenshein, G. E. Interaction of an NF-κB-like factor with a site upstream of the c-myc promoter. Proc. Natl. Acad. Sci. USA, *87:* 4727–4731, 1990.
37. La Rosa, F. A., Pierce, J. W., and Sonenshein, G. E. Differential regulation of the c-*myc* oncogene promoter by the NF-κB rel family of transcription factors. Mol. Cell. Biol., *14:* 1039–1044, 1994.
38. Bird, T. A., Schooley, K., Dower, S. K., Hagen, H., and Virca, G. D. Activation of nuclear transcription factor NF-κB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. J. Biol. Chem., *272:* 32606–32612, 1997.
39. Wang, D., Westerheide, S. D., Hanson, J. L., and Baldwin, A. S., Jr. Tumor necrosis factor α-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. J. Biol. Chem., *275:* 32592–32597, 2000.
40. Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W. R. Phosphorylation of the PTEN tail regulates protein stability and function. Mol. Cell. Biol., *20:* 5010–5018, 2000.
41. Torres, J., and Pulido, R. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. J. Biol. Chem., *276:* 993–998, 2001.
42. Miller, S. J., Lou, D. Y., Seldin, D. C., Lane, W. S., and Neel, B. G. Direct identification of PTEN phosphorylation sites. FEBS Lett., *528:* 145–153, 2002.
43. Faust, R. A., Tawfic, S., Davis, A. T., Bubash, L. A., and Ahmed, K. Antisense oligonucleotides against protein kinase CK2-α inhibit growth of squamous cell carcinoma of the head and neck *in vitro*. Head Neck, *22:* 341–346, 2000.
44. Rayet, B., and Gelinas, C. Aberrant *rel/nfkb* genes and activity in human cancer. Oncogene, *18:* 6938–6947, 1999.
45. Zhou, B. P., Hu, M. C., Miller, S. A., Yu, Z., Xia, W., Lin, S. Y., and Hung, M. C. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-κB pathway. J. Biol. Chem., *275:* 8027–8031, 2000.
46. Sun, L., and Carpenter, G. Epidermal growth factor activation of NF-κB is mediated through IκBα degradation and intracellular free calcium. Oncogene, *16:* 2095–2102, 1998.
47. Finco, T. S., Westwick, J. K., Norris, J. L., Beg, A. A., Der, C. J., and Baldwin, A. S., Jr. Oncogenic Ha-*Ras*-induced signaling activates NF-κB transcriptional activity, which is required for cellular transformation. J. Biol. Chem., *272:* 24113–24116, 1997.
48. Arsura, M., Mercurio, F., Oliver, A. L., Thorgeirsson, S. S., and Sonenshein, G. E. Role of the IκB kinase complex in oncogenic *Ras*- and *Raf*-mediated transformation of rat liver epithelial cells. Mol. Cell. Biol., *20:* 5381–5391, 2000.
49. Tawfic, S., Yu, S., Wang, H., Faust, R., Davis, A., and Ahmed, K. Protein kinase CK2 signal in neoplasia. Histol. Histopathol., *16:* 573–582, 2001.

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Mouse Mammary Tumor Virus c-*rel* Transgenic Mice Develop Mammary Tumors

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Amplification, overexpression, or rearrangement of the c-*rel* gene, encoding the c-Rel NF-κB subunit, has been reported in solid and hematopoietic malignancies. For example, many primary human breast cancer tissue samples express high levels of nuclear c-Rel. While the Rev-T oncogene v-*rel* causes tumors in birds, the ability of c-Rel to transform in vivo has not been demonstrated. To directly test the role of c-Rel in breast tumorigenesis, mice were generated in which overexpression of mouse c-*rel* cDNA was driven by the hormone-responsive mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter, and four founder lines identified. In the first cycle of pregnancy, the expression of transgenic c-*rel* mRNA was observed, and levels of c-Rel protein were increased in the mammary gland. Importantly, 31.6% of mice developed one or more mammary tumors at an average age of 19.9 months. Mammary tumors were of diverse histology and expressed increased levels of nuclear NF-κB. Analysis of the composition of NF-κB complexes in the tumors revealed aberrant nuclear expression of multiple subunits, including c-Rel, p50, p52, RelA, RelB, and the Bcl-3 protein, as observed previously in human primary breast cancers. Expression of the cancer-related NF-κB target genes *cyclin D1*, c-*myc*, and *bcl-xl* was significantly increased in grossly normal transgenic mammary glands starting the first cycle of pregnancy and increased further in mammary carcinomas compared to mammary glands from wild-type mice or virgin transgenic mice. In transient transfection analysis in untransformed breast epithelial cells, c-Rel-p52 or -p50 heterodimers either potently or modestly induced cyclin D1 promoter activity, respectively. Lastly, stable overexpression of c-Rel resulted in increased cyclin D1 and NF-κB p52 and p50 subunit protein levels. These results indicate for the first time that dysregulated expression of c-Rel, as observed in breast cancers, is capable of contributing to mammary tumorigenesis.

Nuclear factor (NF)-κB/Rel is a structurally and evolutionary conserved family of transcription factors distinguished by the presence of a 300 amino acid region, termed the Rel homology domain (RHD), based on its homology with v-Rel, the transforming protein encoded by the RevT avian retrovirus. The RHD is responsible for DNA-binding, dimerization, nuclear translocation, and binding of Rel factors to the IκB inhibitory proteins (reviewed in reference 21). Mammals express five NF-κB members that belong to two classes. The first class includes c-Rel, RelB, and RelA (p65), which are synthesized as mature products and contain a C-terminal transactivation domain. The second class consists of NF-κB1 and NF-κB2, which are synthesized as longer precursors, p105 and p100. Those proteins require C-terminal proteolytic processing to produce the mature p50 and p52 subunits, respectively, which contain the RHD but lack a transactivation domain. Although, both p50 and p52 have been found to transactivate when in association with Bcl-3 protein (7, 20). In most untransformed cells, other than B lymphocytes, NF-κB complexes are sequestered in the cytoplasm bound to specific inhibitory proteins, of which IκB-α is the paradigm. Activation of NF-κB involves phosphorylation and rapid degradation of IκB, allowing for translocation of free NF-κB to the nucleus, where it controls genes involved in cell growth and survival, adhesion, and immune and inflammatory responses, including *cyclin D1*, c-*myc*, and *bcl-xl* (reviewed in references 47 and 56).

Evidence from several laboratories has suggested NF-κB is critically involved in regulation of tumorigenesis. We and others demonstrated aberrant constitutive activation of NF-κB factors in breast cancer (43, 59). High levels of nuclear NF-κB were found in human breast tumor cell lines, carcinogen-transformed mammary epithelial cells, and the majority of primary human and rodent breast tumor tissue samples. Accelerated degradation of the IκB-α inhibitory protein was observed (34), suggesting aberrant regulation of nuclear translocation in breast cancer cells. Inhibition of the constitutive NF-κB activity in human breast cancer cell lines induced apoptosis (59) or led to reduced tumorigenicity (50). Conversely, ectopic expression of c-Rel resulted in resistance to TGF-β-mediated inhibition of proliferation (58). Interestingly, we observed that 21 out of 25 primary human breast cancer tissues examined expressed high levels of nuclear c-Rel (59); similar observations were made by Cogswell and coworkers (15). The overexpression of c-Rel has been implicated in other hematopoietic and solid malignancies as well. For example, c-*rel* gene amplification was seen in ~20% of non-Hodgkin's B-cell lymphomas, including diffuse large-B-cell lymphomas (DBCL) (reviewed in reference 47). In addition, the c-*rel* gene was also found rearranged or overexpressed in some follicular lymphomas and

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DBCL. The higher level of expression of c-Rel, plus RelA, in the activated B-cell (ABC) form of DBCL, was found associated with poorer prognosis (2). Furthermore, inhibition of c-Rel induced apoptosis in immature B-cell lymphomas (66).

The c-*rel* gene encodes a 68-kDa protein which is active mostly in lymphocytes and monocytic, granulocytic, and erythroid cells. Mice lacking c-*rel* are viable but show severely impaired lymphocyte proliferation and immune function, with impaired interleukin-2 expression (36). The X-ray crystal structure of the c-Rel homodimer bound to a DNA target site was resolved recently (28). It confirmed that c-Rel homodimers recognize a different set of κB element DNA sequences compared to c-Rel heterodimers or p50-containing dimers, suggesting that those complexes may have a different range of target genes. The v-*rel* gene, carried by the highly oncogenic avian reticuloendotheliosis virus strain T (Rev-T), is able to cause tumors in birds and transgenic animal models. The v-Rel oncogenic protein differs from its avian progenitor c-Rel by the presence of multiple mutations that increases its expression, nuclear localization, DNA binding, transactivation capability and neoplastic transformation potential (reviewed in reference 22). The direct role of c-Rel in tumorigenesis is still a matter of debate. Overexpression of either avian or human c-Rel was shown to transform primary chicken lymphoid cells (1, 23), although with lower efficiency than v-Rel. Retroviral delivery of avian c-*rel* in young chickens gave rise to lymphoid tumors, and cell transformation involved selection of a C-terminal deleted c-Rel protein with increased oncogenic activity (27). All these observations led us to test the oncogenic properties of c-Rel in vivo, in the mammary gland. Here, we have constructed a mouse model in which the mouse c-*rel* cDNA was driven by the hormone-dependent mouse mammary tumor virus (MMTV) promoter. MMTV–c-*rel* female mice developed late-onset mammary tumors, which were related to increased levels of expression of the NF-κB growth and survival target genes *cyclin D1*, c-*myc*, and *bcl-xl*. These results provide the first in vivo evidence for a causal role of c-Rel activation in the pathogenesis of breast cancer.

MATERIALS AND METHODS

Isolation of founder MMTV–c-*rel* transgenic mice. The 2.5-kb *Eco*RI/*Hind*III fragment from the pSVSport-c-Rel vector, containing the full-length murine c-*rel* cDNA (kindly provided by T. Gilmore, Boston University, Boston, Mass.), was blunt end ligated into the MMTV-LTR plasmid, containing the MMTV long terminal repeat (MMTV-LTR), which directs expression chiefly to the mammary epithelium, with ras 5′ untranslated sequences provided upstream of the cDNA and a simian virus 40 (SV40) intron and polyadenylation signal downstream (38, 55), yielding the pMMTV–c-*rel* plasmid. The direction of the insert was confirmed by restriction mapping and DNA sequencing at the Molecular Biology Core at Boston University Medical School. Plasmid sequences were removed by restriction digestion at the *Sal*I and *Spe*I sites, leaving the MMTV-LTR sequence, ras 5′ untranslated sequences, c-*rel* cDNA, and the SV40 intron and poly(A) addition signal sequence. The excised transgene construct was gel purified and microinjected into pronuclei of fertilized one-cell zygotes from FVB/N mice. These zygotes were reimplanted into pseudopregnant foster mothers, and the offspring were screened for presence of the transgene by Southern blotting (see below). Carriers were bred to establish four independent transgenic lines. Female transgenic mice were continuously bred to induce transgene expression though activation of the hormone-dependent MMTV-LTR promoter. Mice were monitored biweekly for the appearance of tumors. Mice were housed in a two-way barrier at the Boston University School of Medicine Transgenic mouse facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

DNA analysis. Genomic tail DNA was isolated as described previously (46), samples (10 μg) were digested with *Pst*I, and the resulting fragments were separated on a 0.8% agarose gel and transferred to GeneScreenPlus (DuPont NEN) nylon membranes. DNA was cross-linked to the membrane by UV irradiation (Stratalinker; Stratagene, La Jolla, Calif.) at 0.12 J/cm^2 for 30 s. Hybridization was performed using the 2.2-kb DNA fragment, encompassing the MMTV-LTR promoter and an ~1-kb fragment of the mouse c-*rel* cDNA, released from the pMMTV–c-*rel* plasmid by digestion with *Pst*I as a probe. The DNA was radiolabeled by random priming and used as described previously (35), except that 2.0×10^6 cpm/ml ^{32}P-labeled DNA was employed.

RNA analyses. Frozen breast tissue was pulverized in liquid nitrogen with a mortar and pestle, and total RNA extracted with the Ultraspec-II RNA Isolation System (Biotecx Laboratories Inc., Houston, Tex.). To remove contaminating DNA, RNA samples were digested for 30 min at 37°C with RQ1 RNase free DNase (Promega Corporation, Madison, Wis.), according to the manufacturer's directions. RNA was reextracted and ethanol precipitated. For reverse transcriptase PCR (RT-PCR), 5 μg RNA samples were reverse transcribed with SUPERSCRIPT RNase H-RT in the presence of 200 ng of random primers (all reagents from Invitrogen Life Technologies, Carlsbad, Calif.). For PCR, a 236-bp fragment of the transgene was amplified with a sense oligonucleotide primer from the mouse c-*rel* cDNA coding sequence (5′-GTGACCCCTAAGGGTTTTCTG-3′) and an antisense oligonucleotide from the SV40 poly(A) tail of the vector construct (5′-CCCATTCATAAGTTCCATAG-3′). PCR were performed in a thermal cycler (MJ Research, Watertown, Mass.) by denaturing at 95°C for 45 s, annealing at 46°C for 90 s, and extending at 72°C for 90 s for 35 cycles. Primer pairs specific for the mouse *cyclin D1* gene were 5′-CACAACGCACTTTCTTTCCA-3′ and 5′-GACCAGCCTCTTCCTCCAC-3′, and amplified a 164-bp fragment with an annealing temperature of 55°C. The primer pairs specific for mouse *bcl-xl* were as described previously (52). As a control for RNA quality, a 750-bp fragment of β-actin mRNA was amplified by 25 or 30 PCR cycles with the following primers: 5′-ACCAGTTCGCCATGGATGACGATA-3′ and 5′-AGCTCATAGCTCTTCTCCAGGGAG-3′, which were used with an annealing temperature of 55°C. For radiolabeled PCR, 0.85 μCi of [α-^{32}P]dCTP and [α-^{32}P]dGTP were added in the PCR. For RNase protection assays (RPAs), multiprobe RPA kits (Pharmingen, San Diego, Calif.) were used according to the manufacturer's directions. The identity of the RNase protected bands in the gel were established using the undigested probes as markers and a control RNA for mouse cyclin or apoptosis gene mRNA expression (Pharmingen). For Northern blot analysis, RNA samples (5 to 15 μg) were denatured and separated by electrophoresis in 1.0% formaldehyde agarose gels (16). Probes included a 2.4-kb pM-c-myc54 mouse c-*myc* cDNA (60), the full-length human cyclin D1 cDNA subcloned into the pBPST-R1 vector, kindly provided by R. G. Pestell (Albert Einstein College of Medicine, New York, N.Y.), and a 750-bp fragment of the mouse *bcl-x* cDNA amplified by PCR using the previously described primers (52). Quantitation by scanning densitometry was performed with a KDS1D device (version 2.0; Kodak, New Haven, Conn.).

EMSA. Frozen tissue powders were resuspended in homogenization buffer (1 g/ml) in 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM sucrose, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (5 μg/ml), and aprotinin (5 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). Samples were Dounce homogenized for 20 strokes with a loose pestle and then 20 strokes with a tight pestle. The KCl concentration was then adjusted to 100 mM and the nuclei were washed twice with the homogenization buffer with 100 mM KCl. Nuclear proteins were extracted on ice for 30 min in 2 packed nuclear volumes containing 10 mM HEPES (pH 7.9), 400 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, leupeptin (0.5 μg/ml), and aprotinin (5 μg/ml). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.). The sequence of the URE NF-κB-containing oligonucleotide from the c-*myc* gene is as follows: 5′-GATCCAAGTCCGGGTTTTC*CCC*AACC-3′ (17). The core element is underlined. The Octomer-1 (Oct-1) oligonucleotide has the following sequence: 5′-TGTCGAATGCAAATCACTAGAA-3′. Nuclear extracts samples (5 μg) were subjected to electrophoretic mobility shift analysis (EMSA) as described previously (59). For antibody supershift analysis, the binding reaction was performed in the absence of the probe, the appropriate antibody was added and the mixture incubated for 16 h at 4°C. The probe was then added and the reaction incubated an additional 30 min at 25°C and the complexes resolved by gel electrophoresis, as above. Antibodies used included anti-RelA (C-20), sc-372; anti-c-Rel (N), sc-70; and anti-p50 (NLS), sc-114 (all from Santa Cruz Biotechnology, Santa Cruz, Calif.). In addition, rabbit polysera 1266, 1050, and 1051 specific for c-Rel were kindly provided by N. Rice and M. Ernst (National Cancer Institute, Frederick Md.). Data were quantified by densitometry using a Molecular Dynamics densitometer.

Cell lines and transfection conditions. NMuMG, which is an untransformed, immortalized mouse mammary epithelial cell line, was cultured as described previously (57). MCF-10F is a human mammary epithelial cell line established from a patient with fibrocystic disease, which does not display malignant characteristics (9). The RelA and p50 expression vectors have been described elsewhere (39). The p52 and Bcl-3 expression vectors were kindly provided by U. Siebenlist (National Institutes of Health, Bethesda, Md.). The cyclin D1 promoter constructs CD1 −66 WT-Luc and CD1 −66 Mut-Luc, with wild-type (WT) and mutant NF-κB elements, respectively, were a kind gift of R. G. Pestell (25). For luciferase assays, NMuMG cells were transfected in six-well plates using the Fugene reagent (Roche Diagnostics Corporation, Indianapolis, Ind.), and the SV40 promoter–β-galactosidase (pSV40–β-gal) reporter vector was used to normalize transfection efficiency, as previously described (3). For stable transfectants, MCF-10F cells were transfected in P100 dishes with 10 μg of pBluescript or pSVSport-c-Rel vector along with 1 μg of pGKpuro selection plasmid, selected with puromycin (4 μg/ml; Sigma) for 4 days, and then grown in the presence of puromycin (1 μg/ml).

Immunoblot analysis. Whole-cell extracts (WCEs) were prepared in RIPA buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS, 1% sodium sarcosyl, 0.2 mM PMSF, leupeptin [10 μg/ml], 1 mM DTT). Nuclear extracts were prepared as described above. Samples (40 μg) were separated by electrophoresis in 8% polyacrylamide–SDS gels, transferred to a 0.45-μm-pore-size polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) and subjected to immunoblotting, as described (59). Antibodies included anti-RelB (C-19) sc-226, anti-p52 (K-27) sc-298; Bcl-3 (C-14) sc-185, and Sp1 (PEP 2) sc-59 (all from Santa Cruz Biotechnology) and anti-cyclin D1 monoclonal Ab-3 (Oncogene, Boston, Mass.). Antibodies specific for other NF-κB subunits were as described above.

Histology. Upon necropsy, tumors and other mammary glands, heart, lung, liver, kidney, spleen, and the adrenal gland were removed and immediately fixed in Optimal Fix (American Histology Reagent Co., Lodi, Calif.) and shipped in alcohol. The tissues were processed, embedded in paraffin, and sectioned at a thickness of 7 μm. The sections were mounted on glass slides and stained with hematoxylin and eosin using routine laboratory procedures in the Transgenic Core Pathology Laboratory at the University of California, Davis. Sections were compared with other specimens in the extensive mouse mammary tumor database (http://www-mp.ucdavis.edu/tgmice/firststop.html).

RESULTS

Generation and characterization of MMTV–c-*rel* transgenic mice. To determine the role of c-Rel in mammary tumorigenesis, we generated a mouse model where c-*rel* cDNA was expressed under the control of the MMTV-LTR promoter, an effective vector for expressing oncogenes and for transforming the mammary epithelium (11, 61). A construct containing the full-length mouse c-*rel* cDNA was inserted in a plasmid containing the MMTV-LTR promoter and an SV40 T antigen intron-poly(A) cassette to ensure efficient expression in vivo. The insert c-*rel* DNA was then utilized to generate transgenic mice in the FVB/N mouse strain. Integration of the construct into the genome of potential founders was assessed by Southern blot analysis of tail DNA with c-*rel* cDNA as a probe (Fig. 1A and data not shown). Five founders successfully passed the transgene through the germ line. Founder line 18 had approximately two to three copies of the transgene, while lines 7, 14, and 15 had four to five copies and line 16 had approximately nine copies, as estimated by comparison with bands resulting from hybridization with the endogenous gene on the Southern blots. MMTV–c-*rel* transgenic mice of all founders developed and bred normally. Transgenic females were able to nurse their pups.

To characterize the expression pattern of the c-*rel* transgene, line 14 MMTV–c-*rel* mice or WT FVB/N mice, as control, were bred to activate the MMTV-LTR promoter, which contains hormonally responsive elements activated by progestins and corticosteroids. At day 18.5 of the first pregnancy, total RNA was isolated from the mammary glands and various other organs. RNA samples were subjected to a radiolabeled transgene-specific RT-PCR assay, performed with a 5′ mouse c-*rel* cDNA sense primer and a 3′ SV40 poly(A) antisense primer (Fig. 1B). Expression of c-*rel* transgene mRNA was observed in the mammary gland of line 14, but not the WT mouse, as expected (Fig. 1B, left panel). RNA quality and equal loading was confirmed by analysis of β-actin mRNA expression profiles by RT-PCR (Fig. 1B, bottom panel), as well as in ethidium bromide-stained gels (data not shown). At day 18.5 of preg-

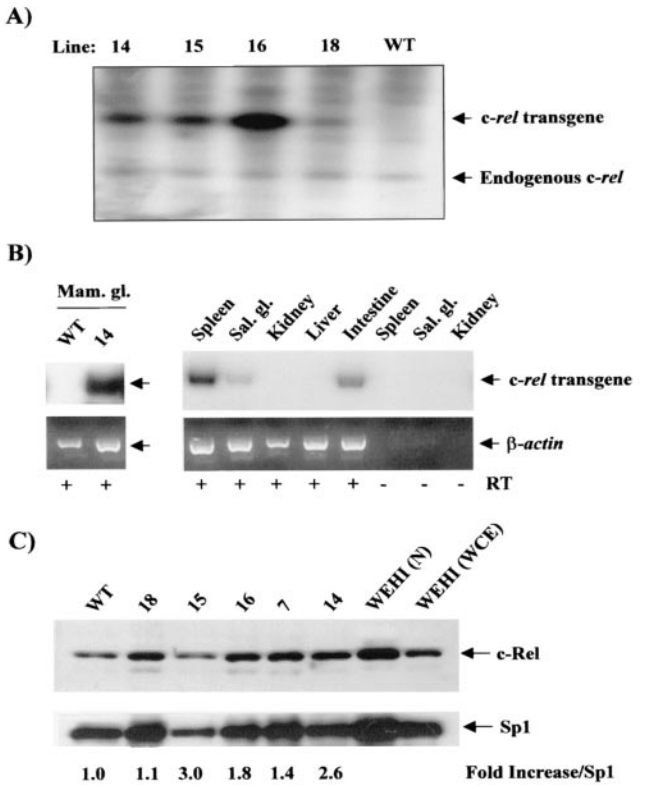

FIG. 1. MMTV-LTR-driven c-*rel* transgene expression in FVB/N mice. (A) Identification of founder lines. Genomic tail DNA was prepared from the indicated potential founders MMTV–c-*rel* transgenic mice, and samples (10 μg) digested with *Pst*I and subjected to Southern blot analysis for c-*rel* using the 2.2-kb fragment, encompassing the MMTV-LTR promoter and an ~1-kb fragment of mouse c-*rel* cDNA, released from the MMTV–c-*rel* plasmid digested with *Pst*I, as a probe. The positions of the bands derived from the c-*rel* transgene and the endogenous c-*rel* gene are as indicated. (B) Transgenic c-*rel* expression. Total RNA was isolated from the indicated organs of WT FVB/N or line14 MMTV–c-*rel* mice at day 18.5 of the first pregnancy, and subjected to DNase treatment. Samples (5 μg) were subjected to RT-PCR analysis, in the presence (+) or absence (−) of RT to control for DNA contamination, using c-*rel* transgene-specific oligonucleotides, amplifying a 236-bp fragment. Similar analysis of β-actin RNA levels confirmed the integrity of the reverse transcription reaction. (C) Total c-Rel expression. Mammary glands were removed from WT FVB/N or the indicated transgenic line mice at day 18.5 of the first pregnancy. Nuclear extract were prepared, and samples (20 μg) subjected to immunoblot analysis of c-Rel, and Sp1, as control for loading. As additional controls, nuclear extracts and WCEs from the WEHI 231 immature B-lymphoma cells, which express high constitutive levels of c-Rel (40), were similarly analyzed. The values of c-Rel normalized to Sp1 level relative to the WT sample are displayed below.

TABLE 1. Tumor incidence and histopathology in female MMTV–c-*rel* transgenic mice after multiple cycles of pregnancy and regression[a]

Mam. tumor diagnosis (other tumors)[b]	Age[c] (mo)	No. of:		Line	Mouse no.[d]
		Mam. tumors	Other tumors		
Mam. squamous cell carcinoma and hyperplasia (bronchial adenocarcinoma)	22	1	1	7	4026
Mam. adenosquamous carcinoma	22	1	0	7	4042
Mam. adenosquamous carcinoma	18	1	0	14	3814
Mam. adenosquamous carcinoma (papillary bronchial adenomas)	20	1	1	14	3983
Mam. adenocarcinomas with pulmonary metastases	23	4	1	14	3996
Mam. papillary carcinoma, lobular hyperplasia, squamous nodules	23	1	0	14	4936
Mam. squamous cell carcinoma and hyperplasia	15	1	0	14	4556
Mam. adenocarcinoma (papillary bronchial adenocarcinomas)	19	1	1	15	127
Mam. squamous cell carcinoma	22	1	0	16	3872
Mam. adenocarcinoma with poorly differentiated large cells	18	1	0	16	3875
Mam. squamous cell carcinoma and hyperplasia (centrocytic lymphoma)	18	1	1	16	4521
Spindle cell tumor, originating from a mam. adenocarcinoma	19	1	0	16	4528

[a] Thirty-eight multiparous female mice from the four transgenic lines (7, 14, 15, and 16) were monitored for tumor incidence by biweekly palpable examination.
[b] Histopathological analysis of mammary (mam.) glands and other organs (heart, lung, liver, kidney, spleen, and adrenal gland) from the same animal was performed when a tumor was detected.
[c] Age when tumor was detected.
[d] An identification number was given to the individual mice of the cohort for further analysis of the tumors.

nancy, expression of transgene c-*rel* mRNA was also observed in the spleen, salivary gland, and intestine of mice of line 14 (Fig. 1B) and line 16 (data not shown), while undetectable or low levels were observed in the kidney and liver (Fig. 1B) and heart and lung (data not shown). Where indicated, reactions were performed in the absence of RT, which confirmed the absence of DNA contamination. Thus, the c-*rel* transgene mRNA is expressed mostly in glandular organs and lymphoid tissues, which is consistent with previous studies with the MMTV-LTR promoter (38, 55).

We next sought to determine total levels of c-Rel protein expression, which includes both endogenous and transgenic c-Rel. At day 18.5 of the first pregnancy, nuclear extracts were prepared from mammary glands of transgenic lines 7, 14, 15, 16, and 18 mice, and from a WT FVB/N mouse as control. Samples were subjected to immunoblot analysis for c-Rel and Sp1 to normalize for loading (Fig. 1C). Nuclei from the WT mammary gland contained basal levels of c-Rel, as has been reported recently (10). All of the transgenic lines displayed higher normalized levels of nuclear c-Rel. The lowest level was seen in line 18, consistent with its low transgene copy number, as seen above; therefore, the other four lines (i.e., lines 7, 14, 15, and 16) were chosen for further study.

MMTV–c-*rel* transgenic mice develop late-onset mammary carcinomas. To promote c-*rel* transgene overexpression, MMTV–c-*rel* female mice were continuously bred to induce the MMTV-LTR promoter. A cohort of 38 multiparous female mice from the 4 expressing lines was monitored for tumor incidence over 2 years. Mice were subjected to biweekly palpable examination, and when the presence of a tumor was detected, the mammary glands and other organs were subjected to histopathological analysis. Thirty-one percent of the mice developed mammary carcinomas at an average age of 19.9 months (Table 1). In contrast, mammary tumors develop with a very low incidence (<1%) in WT female FVB/N mice that have been similarly bred (38). An identification number, with the origin of the line was attributed to each tumor, and characteristics of the different breast tumors are described in the Table 1. Mice from each of the four transgenic lines developed mammary carcinomas. The tumor incidence was 33.3, 41.7, 20, and 33.3% in MMTV–c-*rel* transgenic lines 7, 14, 15, and 16, respectively (data not shown), suggesting that tumor development is related to c-*rel* transgene expression rather than random insertional events. In all but one case, the tumors arose as solitary masses in a single mammary gland. Of the 12 mammary tumors, 3 were pure adenocarcinomas (Fig. 2A), 3 were adenosquamous carcinomas (Fig. 2C), 4 were squamous cell carcinomas (Fig. 2D), 1 was classified as a papillary adenocarcinoma, and 1 was a spindle cell carcinoma (Fig. 2E). Spindle cell carcinomas are often related to an epithelial to mesenchymal cell transition (EMT), which is the transformation of epithelial cells into cells with features of mesenchymal cells, favoring the progression of a carcinoma towards a dedifferentiated and more malignant state (reviewed in reference 63). To test for cells of epithelial origin, immunohistological staining was carried out using antibodies specific for the epithelial cell marker cytokeratin 8 (Fig. 2F). The spindle cell carcinoma stained positively for cytokeratin 8, consistent with an EMT tumor. One of the adenocarcinomas was metastatic to the lung (Fig. 2B).

Lastly, mammary glands of three other multiparous 2-year-old transgenic mice, which were not included in the cohort, were subjected to histopathological analysis even without the presence of a palpable tumor. This examination revealed the presence of an adenosquamous carcinoma and a mammary squamous cell carcinoma in two of the mice (data not shown). In addition to the tumors, poor regression of the alveolar tree of the mammary gland after pregnancy was another histological abnormality that was frequently seen (data not shown). Therefore, the histology of mammary tumors in MMTV–c-*rel* mice appears variable, suggesting changes in mammary epithelial cells during c-Rel-induced tumorigenesis.

c-Rel expression is elevated in mammary glands and tumors of MMTV–c-*rel* transgenic mice. Previous studies showed that the MMTV-LTR promoter is still active in regressing mammary glands, leading to sustained transgene expression over the animal's lifetime (38, 55). We therefore investigated transgenic c-*rel* expression in mammary glands and tumors from

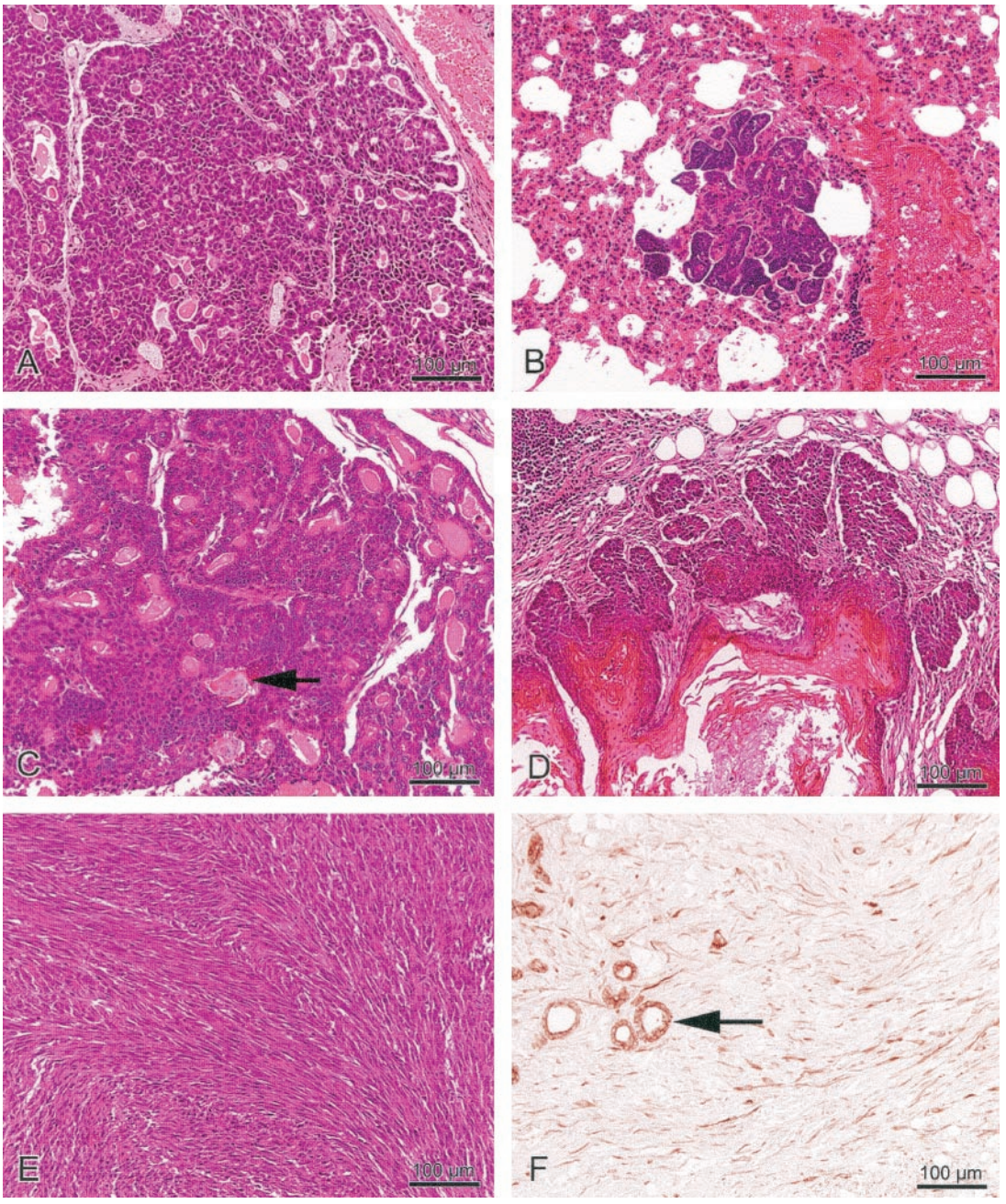

FIG. 2. Representative histopathologies of mammary tumors that developed in MMTV–c-*rel* transgenic mice after multiple cycles of pregnancy and regression. (A) Adenocarcinoma; (B) pulmonary metastasis in a mouse with mammary adenocarcinomas; (C) adenosquamous carcinoma showing areas with extracellular squamous differentiation (arrow); (D) squamous cell carcinoma; (E) spindle cell carcinoma; (F) immunohistochemistry for cytokeratin 8 expression in the spindle cell carcinoma shown in 2E. Note the staining of many of the spindle cells and staining of luminal epithelium in the glands (arrow).

multiparous transgenic mice. RNA was isolated from mammary tumors of mice from transgenic lines 14 and 16 and from grossly normal mammary glands from age-matched transgenic mice (16 to 24 months) that had bred at least three times. In addition, RNA was isolated from mammary glands of a nulliparous (virgin) WT FVB/N mouse and a virgin transgenic line 16 female as negative controls. Samples were subjected to a radiolabeled transgene-specific RT-PCR assay (Fig. 3A). As a control for DNA contamination, reactions were performed in the presence or absence of RT. RNA quality and essentially equal loading was confirmed by analysis of β-actin mRNA expression profiles by RT-PCR (Fig. 3A, bottom panel), and by ethidium bromide staining of gels (data not shown). All tumors and most normal mammary glands of multiparous MMTV–c-*rel* transgenic mice exhibited expression of transgene mRNA, although at variable levels (Fig. 3A, top panel). In tumors, the lowest expression level of transgene mRNA was observed in the mammary tumor developed in mouse 4521 (line 16), whose

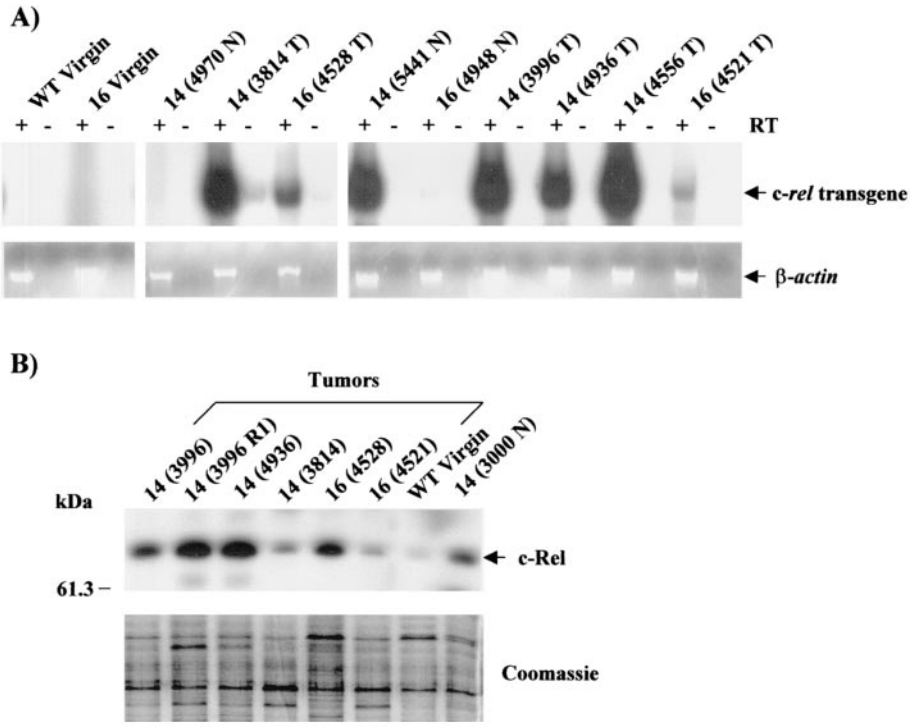

FIG. 3. Expression of c-Rel in transgenic female MMTV–c-*rel* breast tumors. Mammary glands were removed from virgin WT FVB/N (WT Virgin) or transgenic line 16 (16 Virgin) mice and from tumor (T) and grossly normal (N) tissues of multiparous line 14 and 16 transgenic mice. The identification number given to the individual mice is indicated in parentheses. Characteristics of the tumor samples are given in the Table 1. (A) Transgenic c-*rel* expression. RNA was prepared from mammary gland and tumor tissues, and samples subjected to RT-PCR with primers specific for transgenic c-*rel*, as in the legend to Fig. 1. (B) Total protein c-Rel expression. Nuclear extracts were prepared, and samples (40 μg) subjected to immunoblot analysis of c-Rel levels. Coomassie blue staining of SDS-PAGE gels was used as control for equal loading (bottom panel). While the overall levels of staining were essentially equivalent, the analysis revealed that the patterns of protein expression are different between tumors, WT and normal transgenic mammary gland samples. This variability likely results from differences in cell type composition in these tissues.

histologic analysis showed a complex cell pattern, consisting of mammary squamous cell carcinoma and hyperplasic epithelial cells (Table 1). In contrast, virgin WT and transgenic mammary glands did not express detectable levels of transgene mRNA, as expected. Thus, ectopic c-*rel* transgene expression is induced in the mammary gland by pregnancy, and is expressed in the tumors and grossly normal mammary glands of multiparous MMTV–c-*rel* mice.

We next assessed total c-Rel protein expression in mammary glands and tumors of transgenic mice by immunoblot analysis. Nuclear extracts were prepared from the indicated tumors (Fig. 3B). As a control, nuclear extracts were isolated from mammary glands of a WT virgin FVB/N mouse and from nonmalignant mammary glands of an age-related line 14 mouse (3000 N) that had undergone three cycles of pregnancy and regression. The mammary gland of the line 14 mouse (3000 N) and all tumors displayed elevated levels of c-Rel expression compared to the WT mammary gland, which showed only a low basal expression (Fig. 3B and data not shown). This is consistent with the pattern of transgenic c-*rel* expression obtained above. In addition, about half of the tumors showed higher expression levels of c-Rel than grossly normal transgenic mammary glands (Fig. 3B and data not shown). When the results were scanned, a 3- to 150-fold (average, [68.0 ± 60.1]-fold) increase in nuclear c-Rel expression was observed in tumor and normal transgenic mammary glands compared to the WT sample. Equal loading was confirmed by Coomassie blue staining of gels (Fig. 3B, bottom panel), although the patterns of total protein expression appeared different between tumor samples and WT or normal transgenic mammary gland samples. This variability likely results from differences in cell type composition between the various mammary tumors and the normal tissue. Overall, these results indicated that c-*rel* transgenic mRNA and total c-Rel protein expression are upregulated in MMTV–c-*rel* mammary glands and tumors compared to WT mice.

Expression of NF-κB family members in mammary glands and carcinomas of MMTV–c-*rel* transgenic mice. To test for constitutive nuclear c-Rel binding activity, nuclear proteins were isolated from a mammary tumor (adenocarcinoma) and grossly normal mammary glands of line 15 mouse 127 and were subjected to EMSA analysis with an oligonucleotide probe containing the NF-κB element upstream of the c-*myc* promoter, which binds all Rel family members (39) (Fig. 4A). The normal mammary gland displayed a low level of NF-κB binding (better seen on a darker exposure), consistent with our previous findings with nuclear extracts from normal rat mammary glands and histologically normal mammary tissue from MMTV-Her-2/neu transgenic mice (34, 45, 59). The extracts from the c-Rel-induced tumor displayed two major complexes of NF-κB binding. To identify the nature of the subunit components, we used antibodies against either c-Rel or p50 in

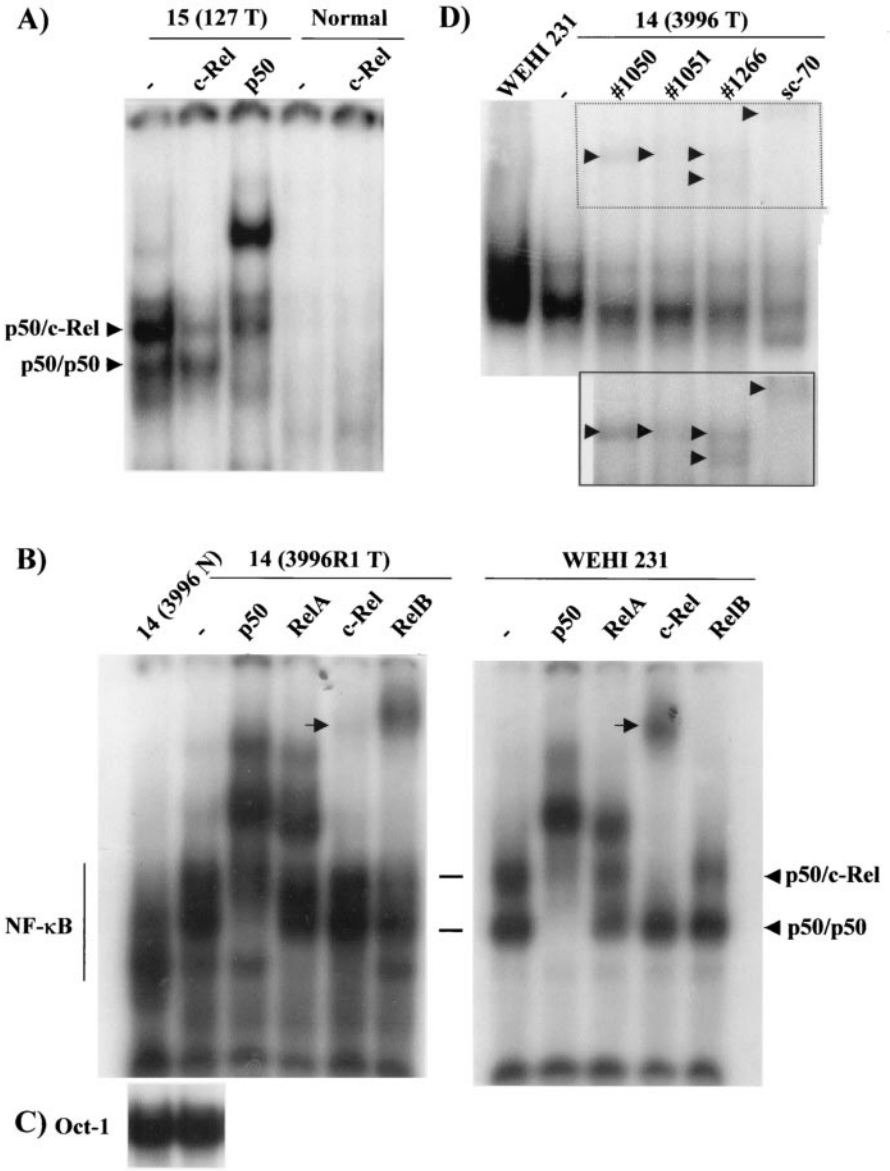

FIG. 4. MMTV–c-*rel* tumors display elevated NF-κB binding. (A) Nuclear extracts were prepared from MMTV–c-*rel* line 15 (127) mouse mammary tumor and grossly normal mammary glands, and samples (5 μg) subjected to EMSA for NF-κB binding. To identify subunit composition, the indicated samples were incubated overnight at 4°C in the absence (−) or the presence of a supershifting antibody specific for p50, or a blocking antibody specific for c-Rel. The positions of the identified p50/c-Rel and p50 homodimer complexes are as indicated. (B and C) Nuclear extracts were prepared from line 14 3996 mammary tumor (3996R1 T) and grossly normal mammary glands (N), and samples (5 μg) were subjected to EMSA for NF-κB (B) and, as a loading control, Oct-1 (C). For supershift analysis, samples were incubated overnight at 4°C in the absence (−) or the presence of supershifting antibodies specific for p50, RelA, c-Rel (sc-70), and RelB. The arrow shows the position of the c-Rel supershifted complex. Where indicated, nuclear extracts (5 μg) of WEHI 231 B cells, which express high levels of c-Rel/p50 complexes (40), were analyzed as a positive control in the same experiment, however a lighter exposure is shown for the WEHI 231 samples. (D) A nuclear extract was prepared from line 14 3996 mammary tumor (3996 T), and samples (5 μg) were subjected to EMSA for NF-κB, with a WEHI 231 nuclear extract, in the absence or presence of the indicated c-Rel-specific antibodies, as above. Inset represents a darker exposure of the region of the supershifted bands indicated in the figure.

EMSA. Addition of an antibody that preferentially recognizes p50 in a homodimer complex, shifted the bottom band completely, and reduced the more slowly migrating complex. Addition of an antibody against c-Rel selectively reduced the top band. These results suggest that the upper complex consisted of p50/c-Rel heterodimers, while the bottom complex was a homodimer of p50.

We next performed EMSA on mammary tumor (adenocarcinoma) samples from a line 14 (3996) mouse. This mouse had developed four mammary adenocarcinomas, as well as pulmonary metastases (Table 1). Nuclear extracts isolated from one of the mammary tumors (3996R1) displayed multiple NF-κB complexes (Fig. 4B). The level of NF-κB binding was more intense than seen with the nuclear extract from a nonmalignant

mammary gland from the same animal (line14 3996 N), while levels of control Oct-1 binding were similar in these samples (Fig. 4C). Of note, an NF-κB complex of higher mobility was present in the normal sample. We previously observed the presence of such a complex in nuclear extracts isolated from normal human and mouse breast tissue, and supershift analysis showed that these complexes contained predominantly p50 homodimers (reference 50 and data not shown). Addition of a c-Rel-specific antibody resulted in diminished binding and the formation of one slowly migrating supershifted complex (Fig. 4B, indicated by an arrowhead). Addition of an antibody against c-Rel reduced the binding and yielded a similar supershifted complex with control extracts from WEHI 231 immature B-lymphoma cells, which express high levels of activated c-Rel and p50, and lower levels of RelA (40, 48), indicating that the line14 3996R1 tumor sample contains p50/c-Rel complexes. Addition of supershifting antibodies against p50, RelA, or RelB to the line 14 3996R1 extracts resulted in shifted complexes. In particular, addition of a p50 antibody greatly reduced binding and yielded two major and a few minor supershifted bands. Interestingly, addition of a RelA antibody yielded three supershifted RelA-containing complexes, whereas, the expected one RelA-containing complex (e.g., p50/RelA) was seen with the WEHI 231 extract (40). These results suggest that RelA is present in multiple complexes in the mammary gland tumor sample. Lastly, a RelB antibody also reduced binding with the line 14 tumor extract, yielding one supershifted complex (Fig. 4B). Addition of the RelB antibody had no detectable effect on the WEHI 231 cells, consistent with the lack of nuclear RelB in these cells (48). To further characterize the c-Rel binding, we compared the effects of the anti-c-Rel sc-70 from Santa Cruz with three different c-Rel antibody rabbit polysera obtained from N. Rice and M. Ernst (sera 1050, 1051, and 1266), using nuclear extracts from a second tumor from line 14 3996. All of the antibodies reduced the upper complex which comigrated with the WEHI 231 c-Rel/p50 band to approximately the same extent (Fig. 4D). The positions of the supershifted complexes varied for all of the antibodies (Fig. 4D, inset). These finding confirm the presence of c-Rel in NF-κB DNA binding complexes, although, it was not the primary component in the nuclear extracts. Together, these results indicate that multiple NF-κB subunits are binding in the tumor samples, including c-Rel, p50, RelA, and RelB.

Mammary tumors contain multiple NF-κB subunits. The finding that nuclear extracts of mammary tumors from the MMTV–c-*rel* mice contain multiple NF-κB complexes led us to more fully assess the nature of the NF-κB subunit expression in mammary glands and tumors. Immunoblot analysis was performed for the p50, RelA, RelB, and p52 NF-κB subunits, and for Bcl-3 protein in nuclear extracts from mammary tumors developed in MMTV–c-*rel* mice, and from uninvolved mammary glands from a multiparous age-related line 14 transgenic mouse (3000 N), and from a WT virgin FVB/N mouse (Fig. 5). Interestingly, expression of the subunits encoded by genes that are regulated by NF-κB, e.g., p50, p52, and RelB (reviewed in reference 44) appeared substantially increased in many of the samples from the transgenic mice. A dramatic increase in expression of p50 was seen in essentially all of the tumor specimens, as well as grossly normal transgenic mammary gland samples compared to the WT sample. Densitometry indicated

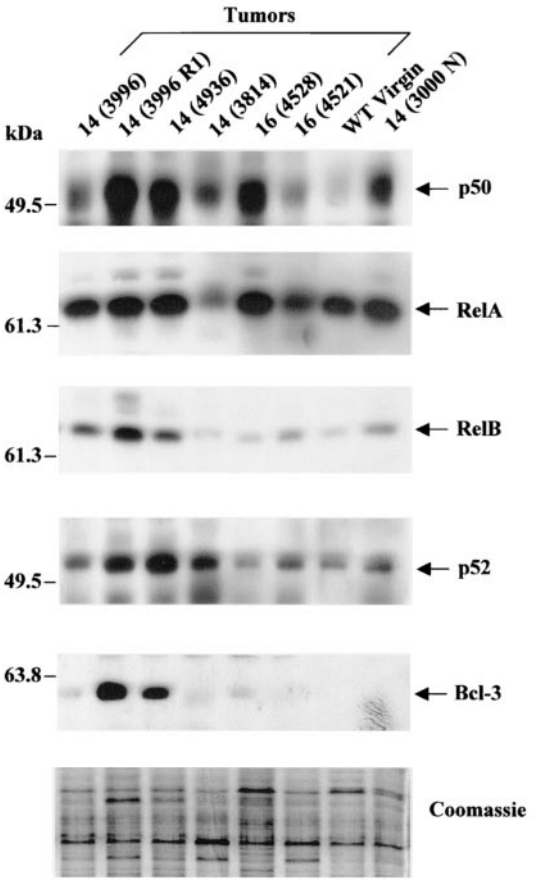

FIG. 5. MMTV–c-*rel* tumors express multiple NF-κB subunits and the Bcl-3 protein. Nuclear extracts were prepared from the indicated mammary tumors, grossly normal mammary glands (N) of multiparous line 14 MMTV–c-*rel* mice, and from mammary glands of a WT nulliparous FVB/N mouse (WT Virgin). Samples (40 μg), subjected to immunoblot analysis as in Fig. 3B, were reprobed for expression of the p50, RelA, RelB, and p52 NF-κB subunits and the Bcl-3 protein. The positions of molecular mass markers are indicated.

a 2.1- to 618-fold induction (average, [330 ± 325]-fold) induction in p50 nuclear levels. Expression of RelB displayed a large increase in three of the specimens compared to the WT mammary gland sample, and a moderate increase in the other 4 samples. In contrast, RelA appeared only moderately increased in most transgenic mammary glands compared to WT mammary glands. Densitometry showed a 1.0- to 1.6-fold increase (average, [1.3 ± 0.4]-fold) in RelA nuclear expression. Interestingly, this contrasted with the relatively high level of RelA binding previously seen in the EMSA of line 14 3996R1 (Fig. 3B), and of other 2 tumor samples that were similarly analyzed (data not shown). Bcl-3 expression was detectable only in tumor samples, and two of them also displayed extremely high levels of p50 and p52 expression. No detectable IκB-α was seen in the nuclear extracts, and no differences were observed in IκB-β levels (data not shown). Overall, these findings indicate that in addition to the c-Rel subunit itself, the MMTV–c-*rel* mammary gland tumors display a wide range of constitutively active nuclear NF-κB subunits, including p50, p52, and RelB, and to a lesser extent RelA, as well as Bcl-3.

Similar complex patterns were seen previously in primary human breast cancer specimens (15, 59).

MMTV–c-*rel* mammary glands and carcinomas display elevated expression of downstream target gene *cyclin D1*. The NF-κB target gene *cyclin D1* (25, 26) has been implicated in breast cancer (reviewed in reference 32). Its expression, which is upregulated in ~50% of human breast tumors, is required for proliferation of breast cancer cells in culture, and MMTV-*cyclin D1* mice develop mammary adenocarcinomas (64). Therefore, we sought to test whether cyclin D1 mRNA levels were increased during pregnancy in the mammary glands of MMTV–c-*rel* versus WT mice. Lines 7, 14, 15, and 16 MMTV–c-*rel* mice and age-matched WT FVB/N mice (3 to 6 months old) were bred to activate the MMTV-LTR promoter in transgenic mammary glands. At day 18.5 of the first pregnancy, total RNA was isolated from the mammary glands and samples subjected to analysis of cyclin D1 mRNA levels using either a semiquantitative RT-PCR assay specific for cyclin D1 in the presence or absence of RT, as control, or Northern blot analysis (Fig. 6A). For the RT-PCR assay, RNA quality and loading were normalized by evaluation of β-actin levels using 25 cycles of PCR, which is within the linear phase of amplification. The level of cyclin D1 mRNA was higher in all of the transgenic mouse mammary glands compared with the three WT mouse samples. When results of this and a duplicate experiment were scanned and normalized to β-actin mRNA levels, a (2.5 ± 0.8)-fold increase ($P < 0.003$) in cyclin D1 mRNA levels was observed in transgenic compared to WT samples. Similarly, higher levels of cyclin D1 mRNA were observed in the transgenic mouse mammary glands compared with WT mouse samples in the Northern blot analysis. RNA loading was normalized to the levels of 28S rRNA. Expression of cyclin D1 mRNA could be detected in two of the five WT mice tested (WT 3 and WT 14). All transgenic mice displayed detectable expression levels of cyclin D1 mRNA, and these levels were higher than those of the WT 3 and WT 14 samples. When results were scanned and normalized to 28S rRNA levels, a (6.8 ± 5.4)-fold increase in cyclin D1 mRNA levels were observed in the transgenic samples compared to WT 3 and WT 14 samples. Thus, overexpression of transgenic c-Rel in the mammary gland during the first pregnancy is sufficient to induce a substantial increase in cyclin D1 mRNA expression.

We next evaluated cyclin D1 levels in c-Rel-induced mammary tumors compared to nonmalignant mammary glands in transgenic animals, again using both the semiquantitative RT-PCR assay and Northern blot analyses (Fig. 6B). Total RNA was isolated from mammary carcinomas that had developed in transgenic mice of lines 14 and 16, as well as from grossly normal mammary glands from multiparous age-related transgenic mice of the same lines. As a reference for basal levels of cyclin D1 mRNA in the mammary gland, total RNA was extracted from mammary glands of five virgin adult transgenic mice of lines 16 or 15. In this and a duplicate RT-PCR assay, all of the tumors displayed higher expression levels of cyclin D1 compared to the two grossly normal mammary gland samples tested. Interestingly, the normal sample 14 (5441 N) displayed higher levels of cyclin D1 mRNA than the normal sample 16 (4948 N), which correlated with their respective levels of c-*rel* transgene expression as seen above (Fig. 3A). In Northern blot analysis, levels of cyclin D1 mRNA were barely detectable in the five virgin mammary gland samples tested. In contrast, one (14 4949 N) of the three grossly normal mammary gland samples and one tumor (14 3814 T) displayed detectable expression levels of cyclin D1 mRNA, while the cyclin D1 mRNA levels were sharply increased in the 3 other tumors (16 4528 T, 14 3996 T, and 14 4936 T). Therefore, MMTV–c-*rel* mammary glands and carcinomas display a substantial overexpression of cyclin D1 mRNA compared to the WT mammary gland.

Evidence has also suggested NF-κB mediates regulation of *cyclin A* (reviewed in reference 32). Thus, we compared the overall cyclin expression profiles in the c-Rel-induced mammary tumors with the grossly normal mammary glands. In the first set, total RNA was prepared from two grossly normal mammary glands from multiparous transgenic mice line 14 (4949 N and 4946 N), and two mammary tumors of line 14 (3814 T) and 16 (4528 T). In a second set, total RNA was prepared from mammary carcinomas of line 14 (4936 T and 4556 T) and line 16 (4521 T). RNA samples were subjected to a multiprobe RPA kit, which assesses mRNA levels for cyclins A1, A2, B1, B2, C, D1, D2, and D3, and L32 and GAPDH housekeeping gene products (Fig. 7). In this and a duplicate experiment, bands were detectable for cyclin A2, B1, D1, D2, and D3 mRNA. The mRNA from the c-Rel-induced tumors displayed increased expression of the *cyclin D1* gene compared to the normal mammary glands (Fig. 7, left panel), consistent with the Northern blot analysis above (Fig. 6B). The tumors displayed variable levels of mRNA for cyclins A2, B1, D2, and D3. Analysis of the housekeeping genes *L32* and *GAPDH* confirmed the essentially equal loading of samples within the panels. Thus, RPA showed that MMTV–c-*rel* tumors display an increase in *cyclin D1* gene expression with variable levels of expression of the other cyclins.

MMTV–c-*rel* mammary tumors overexpress *c-myc*. We next tested for changes in *c-myc* gene expression, another NF-κB target gene (17, 39), which affects cell proliferation and survival (reviewed in reference 5). RNA was isolated at day 18.5 of the first pregnancy from mammary glands of line 7, 15, and 16 MMTV–c-*rel* transgenic and WT FVB/N mice, all 3 to 6 months old. Samples were subjected to Northern blot analysis for c-*myc* RNA levels (Fig. 8A). The quality of the RNA was evaluated by ethidium bromide staining of the gel (Fig. 8A) and by Northern blot analysis of GAPDH mRNA expression levels (data not shown). The level of c-*myc* mRNA appeared higher in most of the mammary glands of the transgenic animals compared to the two WT mice. When results were scanned and normalized to 28S rRNA levels, a (2.4 ± 1.4)-fold increase was observed in c-*myc* mRNA expression levels in transgenic samples compared to the average of the WT samples. Comparable increase was obtained upon normalization to levels of GAPDH mRNA ([2.8 ± 0.7]-fold, data not shown). These levels of increase did not reach statistical significance.

We next compared the levels of c-*myc* mRNA expression in mammary glands versus carcinomas of multiparous age-related transgenic mice and a virgin transgenic mouse. Quality of the RNA and equal loading was checked by ethidium bromide staining of the gel (Fig. 8B) and by RT-PCR analysis of β-actin mRNA levels, as shown above in Fig. 3B (bottom panel). In Northern blot analysis, all the normal and tumor mammary glands of c-Rel-expressing multiparous transgenic mice demonstrated a substantial increase in c-*myc* mRNA expression

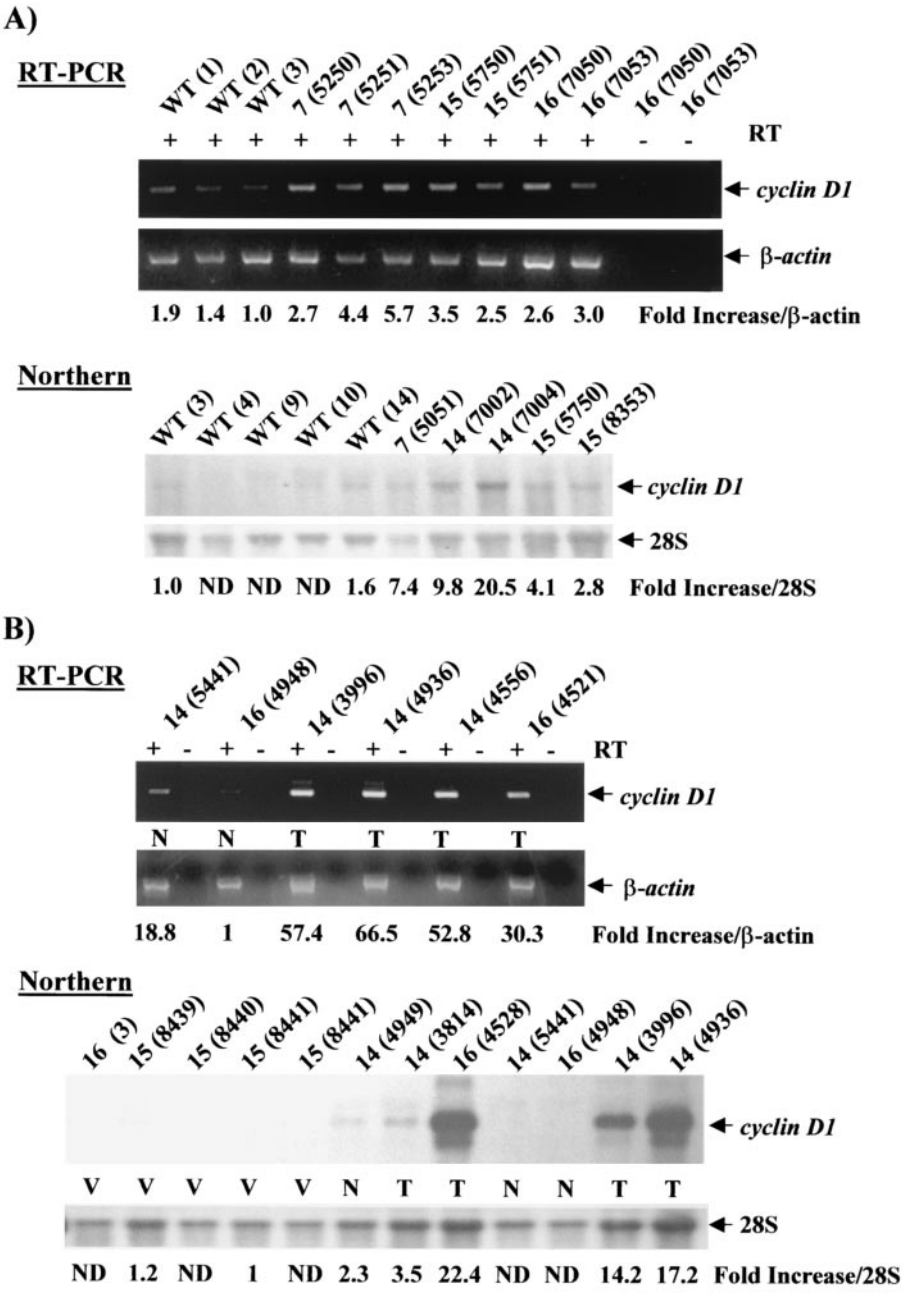

FIG. 6. MMTV–c-*rel* mammary glands and carcinomas overexpress cyclin D1 mRNA. (A) Mammary glands. Total RNA was prepared from mammary glands of 3- to 6-month-old mice or WT FVB/N mice at day 18.5 of the first pregnancy. RT-PCR: RNA was subjected to DNase treatment, and analysis on ethidium bromide stained gels verified quality and essentially equal loading (data not shown). Samples (5 μg) were subjected to RT-PCR analysis of cyclin D1 and β-actin mRNA levels in the presence (+) or absence (−) of RT to control for DNA contamination. For the β-actin mRNA analysis, levels appeared saturated after 30 cycles of PCR and identical in all of the samples tested, while levels were undetectable below 20 PCR cycles (data not shown), so 25 PCR cycles was selected for normalization. The values of cyclin D1 signal intensity normalized to β-actin mRNA levels are presented relative to the WT (3) sample. RNA samples (5 μg) were subjected to Northern blot analysis of cyclin D1 gene levels using a radiolabeled human full-length cyclin D1 cDNA as probe. Ethidium bromide staining of the 28S rRNA was used as a control for loading. The values of cyclin D1 signal normalized to 28S rRNA relative to WT (3) are given below. ND, not detectable by scanning. (B) Mammary carcinomas. Total RNA was prepared from the indicated mammary tumors (T) and grossly normal mammary glands (N) of age-related multiparous line 14 and 16 MMTV–c-*rel* mice, as well as from virgin (V) transgenic mouse mammary glands. RNA samples (5 μg) were subjected to semiquantitative RT-PCR analysis to assess cyclin D1 mRNA levels, as described above. The values of cyclin D1 signal intensity normalized to β-actin RNA levels are presented relative to the line 16 (4948 N) normal sample (which was better seen on a darker exposure). RNA samples (5 μg) were subjected to Northern blot analysis to assess cyclin D1 mRNA levels, as described above. The values of cyclin D1 signal normalized to 28S rRNA relative to 15 (8441) are given below. ND, not detectable by scanning.

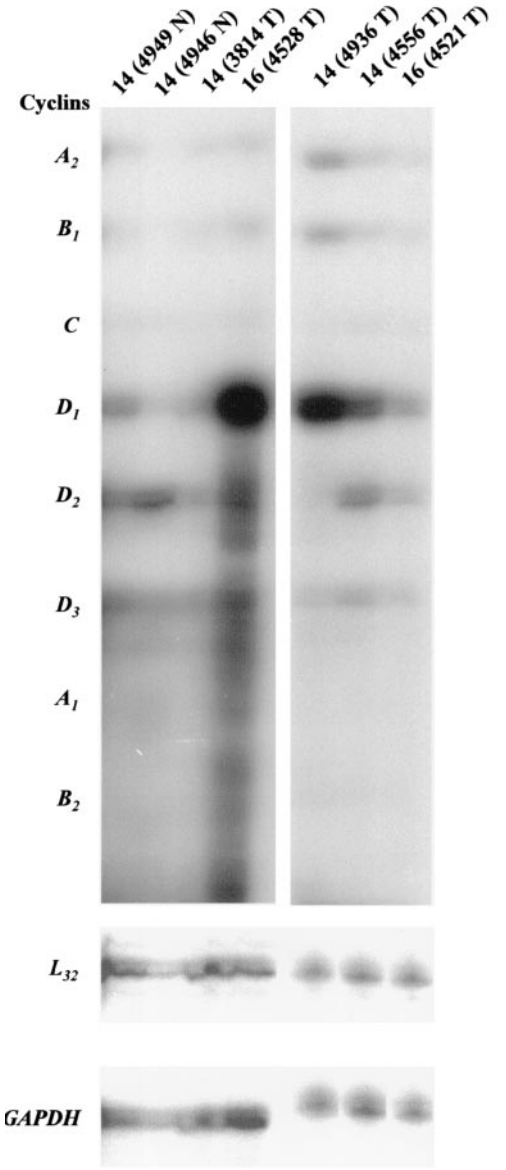

FIG. 7. Profile of cyclin gene expression in MMTV–c-*rel* mouse mammary glands and tumors. Total RNA was prepared from the indicated mammary tumors (T) or grossly normal mammary glands (N) of age-related multiparous line 14 and 16 MMTV–c-*rel* mice. RNA samples (5 μg) were subjected to RPA analysis to assess mRNA levels for cyclin A1, A2, B1, B2, C, D1, D2, and D3, and L32 and GAPDH housekeeping genes. Data from two sets of analyses are shown in the left and right panels. The identities of the RNase protected bands were established using the undigested probes as markers and a control RNA for mouse *cyclin* mRNA expression provided with the kit (data not shown).

levels compared to the line 16 virgin mouse sample. When the results were scanned and normalized to levels of 28S rRNA, a (16.5 ± 8.3)-fold increase in c-*myc* mRNA expression levels was observed in mammary glands of multiparous transgenic mice compared to the virgin transgenic mouse sample. In addition, tumors displayed a (2.3 ± 0.8)-fold increase in levels of c-*myc* RNA expression compared to grossly normal mammary glands of multiparous MMTV–c-*rel* transgenic mice ($P <$ 0.028) (Fig. 8B). Thus, increased c-*rel* expression in transgenic mice leads to elevated levels of c-*myc* mRNA in mammary glands and carcinomas.

MMTV–c-*rel* mammary glands and tumors overexpress *bcl-xl* RNA. The *bcl-xl*, and *bfl-1*/*a1* genes, which encode two prosurvival members of the Bcl-2 family, have been identified as direct targets of NF-κB (13, 70). Bcl-2 expression has also been found to be upregulated by NF-κB (12, 30, 37), but it is unclear if this is a direct effect of NF-κB. Given the evidence for the role of the Bcl-2 family of cell death regulators in mammary tumorigenesis (29), we next assessed mRNA levels of Bcl-2 family members using a second RPA kit. This kit measures RNA levels of the prosurvival genes *bcl-2*, *bcl-xl*, and *bfl-1*/*a1*, and of *bax*, *bak*, and *bad*, which promote apoptosis. RNA samples, isolated from two tumors and two grossly normal mammary glands of MMTV-transgenic mice were subjected to RPA (Fig. 9). Results of two independent experiments showed an increase in the expression of *bcl-xl* RNA levels in the two tumors tested compared to normal mammary glands. When results were scanned and normalized to L_{32} mRNA levels, an ~6.6- and 3.2-fold increase in *bcl-xl* mRNA expression levels were observed in tumor samples 14 (3814 T) and 16 (4528 T), respectively, compared to the two normal

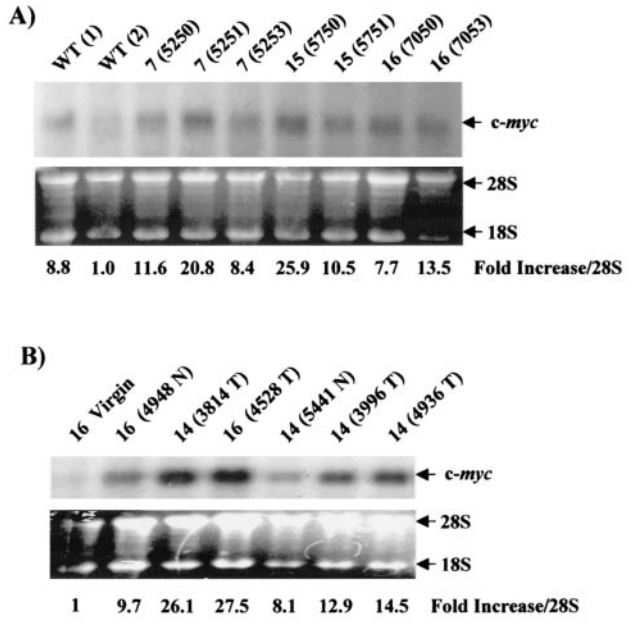

FIG. 8. MMTV–c-*rel* tumors overexpress c-*myc* mRNA. (A) Mammary glands. Total RNA was prepared from mammary glands and samples (15 μg) subjected to Northern analysis for c-*myc* mRNA expression. As a control, the gel was stained with ethidium bromide, shown below. The relative values of c-*myc* signal intensity normalized to levels of 28S rRNA are given relative to the WT (2) virgin sample. (B) Mammary tumors. Total RNA was extracted from the indicated mammary tumors (T) and grossly normal mammary glands (N) of age-related multiparous line 14 and 16 MMTV–c-*rel* mice. In addition, RNA was isolated from mammary glands of a nulliparous transgenic line 16 mouse (16 Virgin). RNA samples (20 μg) were subjected to Northern analysis for c-*myc* mRNA expression. As controls for RNA integrity and equal loading, the gel was stained with ethidium bromide, and RNA samples subjected to RT-PCR analysis for β-actin mRNA levels (Fig. 3). The values of c-*myc* signal intensity normalized to 28S rRNA levels relative to the line 16 virgin sample are given below.

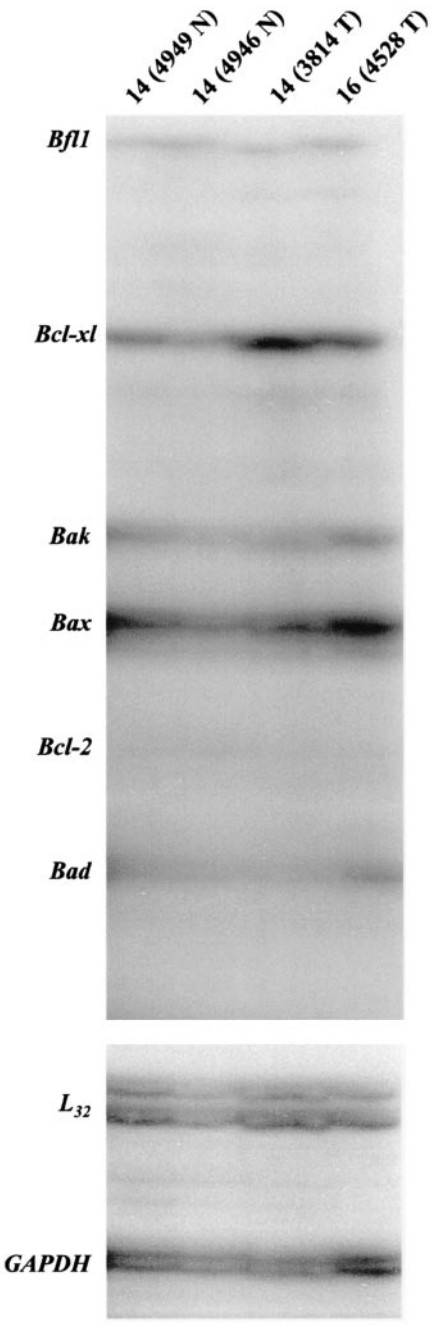

FIG. 9. Profile of Bcl-2 family member gene expression in MMTV–c-*rel* mouse mammary glands and tumors. RNA was prepared from the indicated mammary tumors (T) and grossly normal mammary glands (N) of age-related multiparous line 14 and 16 MMTV–c-*rel* mice. Samples (5 μg) were subjected to RPA analysis to assess mRNA levels of Bcl-2 family member genes, i.e., *bfl-1*/*a1*, *bcl-xl*, *bax*, *bak*, *bcl-2*, and *bad*, and L32 and GAPDH housekeeping genes. The identity of the RNase protected bands were established using the undigested probes as markers and a control RNA for mouse apoptosis gene expression provided with the kit (data not shown).

samples. By contrast, only low and unchanged expression of *bcl-2* and *bfl-1*/*a1*, respectively, was observed. The mRNA expression of the pro-apoptotic genes *bax*, *bak*, and *bad* appeared to change only modestly in the tumor samples. Essentially equal loading was confirmed by analysis of the *L32* and *GAPDH* housekeeping genes. These results suggest that c-Rel-induced mammary tumors display increased levels of *bcl-xl* mRNA expression.

To further study the involvement of Bcl-x_L in c-Rel-induced mammary tumorigenesis, we measured *bcl-xl* mRNA expression levels in tumors as well as during pregnancy in the mammary glands of MMTV–c-*rel* versus WT mice. Samples of total RNA isolated from the mammary glands at day 18.5 of the first pregnancy of lines 7, 14, 15, and 16 MMTV–c-*rel* mice and age-matched WT FVB/N mice, described above, were subjected to semiquantitative RT-PCR and to Northern blot assays of *bcl-xl* mRNA levels (Fig. 10A). For the RT-PCR, RNA quality and loading were normalized to β-actin levels, as described above in Fig. 6. The level of *bcl-xl* mRNA was higher in mammary gland samples from all of the transgenic mice compared with those from the three WT mice. When results of this and a duplicate experiment were scanned a (3.0 ± 0.8)-fold increase ($P < 0.02$) in normalized *bcl-xl* mRNA levels was observed in transgenic compared to WT mammary gland samples. In the Northern blotting, RNA quality and loading were normalized to the levels of 28S rRNA (Fig. 10A), as described above. The RNA band detected with the probe comigrated with the transcript seen in NIH 3T3 cells transiently transfected with a human *bcl-xl* plasmid expression vector. Thus, the transcript is likely *bcl-xl* rather than the *bcl-xs* mRNA. In the 5 WT samples tested, *bcl-xl* mRNA levels were barely detectable, while 4 out of 5 transgenic samples displayed detectable levels of *bcl-xl* mRNA. Taken together, these findings indicate that expression of *bcl-xl* mRNA increases by day 18.5 of the first pregnancy in the mammary glands of c-*rel* transgene mice.

To further study the involvement of Bcl-x_L in c-Rel-induced mammary tumorigenesis, levels of *bcl-x1* mRNA levels were compared in samples of total RNA isolated from mammary carcinomas, grossly normal mammary glands from multiparous age-related transgenic mice, as well as from virgin mice (Fig. 10B). As judged by RT-PCR, all of the tumors displayed higher levels of *bcl-xl* mRNA expression compared to the normal samples tested, in this and a duplicate experiment. Interestingly, once again the normal line 14 (5441 N) displayed higher levels of *bcl-xl* mRNA than the normal sample 16 (4948 N), which correlated with their respective levels of c-*rel* transgene and cyclin D1 mRNA expression seen above (Fig. 3A and 8B). Expression levels of *bcl-xl* mRNA in all virgin mammary glands as well as all grossly normal mammary glands from multiparous transgenic mice were below levels of detection by Northern blot. In contrast, expression of *bcl-xl* mRNA was readily detected in the four tumors tested. Altogether, these results indicate that enforced expression of c-Rel in mammary epithelial cells leads to increased mRNA levels of *cyclin D1*, c-*myc*, as well as *bcl-xl* in the mammary glands and derived tumors.

c-Rel-p52 and p50 heterodimer complexes induce cyclin D1 promoter activity in mammary epithelial cells. While the c-*myc* and *bcl-xl* genes have been shown to be direct targets of c-Rel (13, 24, 39, 70), the cyclin D1 promoter has been shown to be activated by classical p50/RelA complexes (25, 26), and even more potently by Bcl-3/p52 (65). Since no studies have fully investigated the role of c-Rel in the regulation of the cyclin D1 promoter, we compared the activation of the cyclin D1 promoter by different NF-κB complexes in mammary epi-

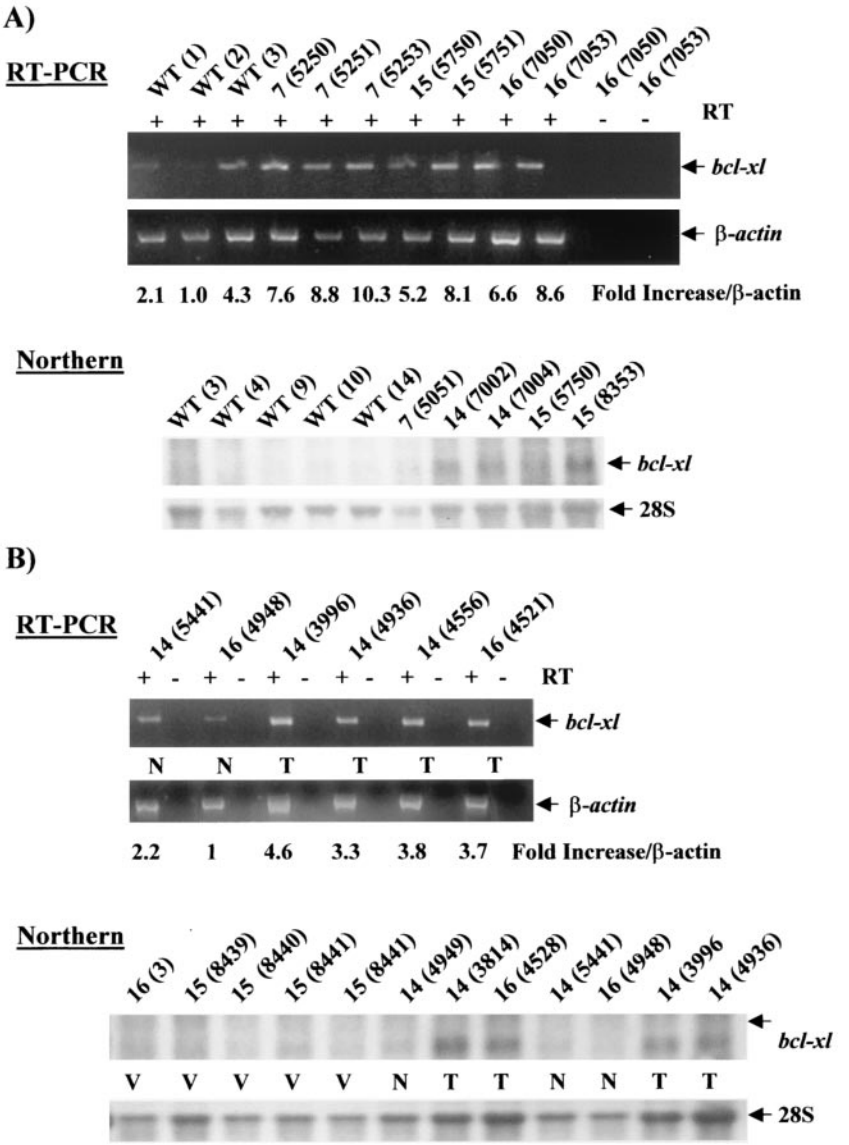

FIG. 10. MMTV–c-*rel* mammary glands and carcinomas overexpress *bcl-xl* mRNA. (A) Mammary glands. Total RNA was prepared from the indicated 3- to 6-month-old transgenic mice and WT FVB/N at day 18.5 of the first pregnancy. RT-PCR: Samples (5 μg) were subjected to RT-PCR analysis of *bcl-xl* and β-actin mRNA levels, as described above in Fig. 6. The values of *bcl-xl* signal intensity normalized to β-actin mRNA levels are presented relative to the WT (2) sample. Northern blot: Samples (5 μg) were subjected to Northern blot analysis of *bcl-xl* mRNA levels, using a mouse *bcl-xl* cDNA as a probe, as described above in Fig. 6. The values of the *bcl-xl* signals for the WT samples were below the level of detection. (B) Mammary carcinomas. Total RNA was prepared from the indicated mammary glands (virgin [V] or multiparous grossly normal [N]) or tumors (T), and samples (5 μg) were subjected to either semiquantitative RT-PCR or Northern blot analysis to assess mRNA levels for *bcl-xl*, as described above. The values of *bcl-xl* signal intensity normalized to β-actin mRNA levels in the RT-PCR analysis are presented relative to the line 16 (4948) normal sample, while the *bcl-xl* signals in the Northern blot analysis for the virgin and normal samples were below the level of detection.

thelial cells. NMuMG mouse untransformed mammary epithelial cells were transfected with vectors expressing cyclin D1 promoter luciferase reporter constructs containing either a wt (−66 wt) or mutated (−66 mut) proximal NF-κB element, plus pSV40-β-gal, for normalization, in the absence or presence of various NF-κB expression vectors (Fig. 11). Expression of p50 or p52 alone results in only a modest increase in cyclin D1 promoter activity. Consistent with previous reports (65), coexpression of p52 and Bcl-3 resulted in a sharp increase in cyclin D1 promoter activity, while p50 and Bcl-3 complexes were less potent. At the higher dose tested, RelA plus p50 caused an increase in cyclin D1 promoter activity. Interestingly, coexpression of c-Rel and p52 resulted in an activation of the cyclin D1 promoter activity comparable to that seen with p52 plus Bcl-3, whereas c-Rel/p50 complexes were again less potent. As controls for protein expression levels, NIH 3T3 cells were similarly transfected, and similar 5 to 10-fold increases in levels of c-Rel, RelA, p50, p52, and Bcl-3 were observed compared to cells transfected with the empty vector DNA (data not shown). These findings indicate that c-Rel can potently induce the cyclin D1 promoter, in particular when present with the p52 subunit.

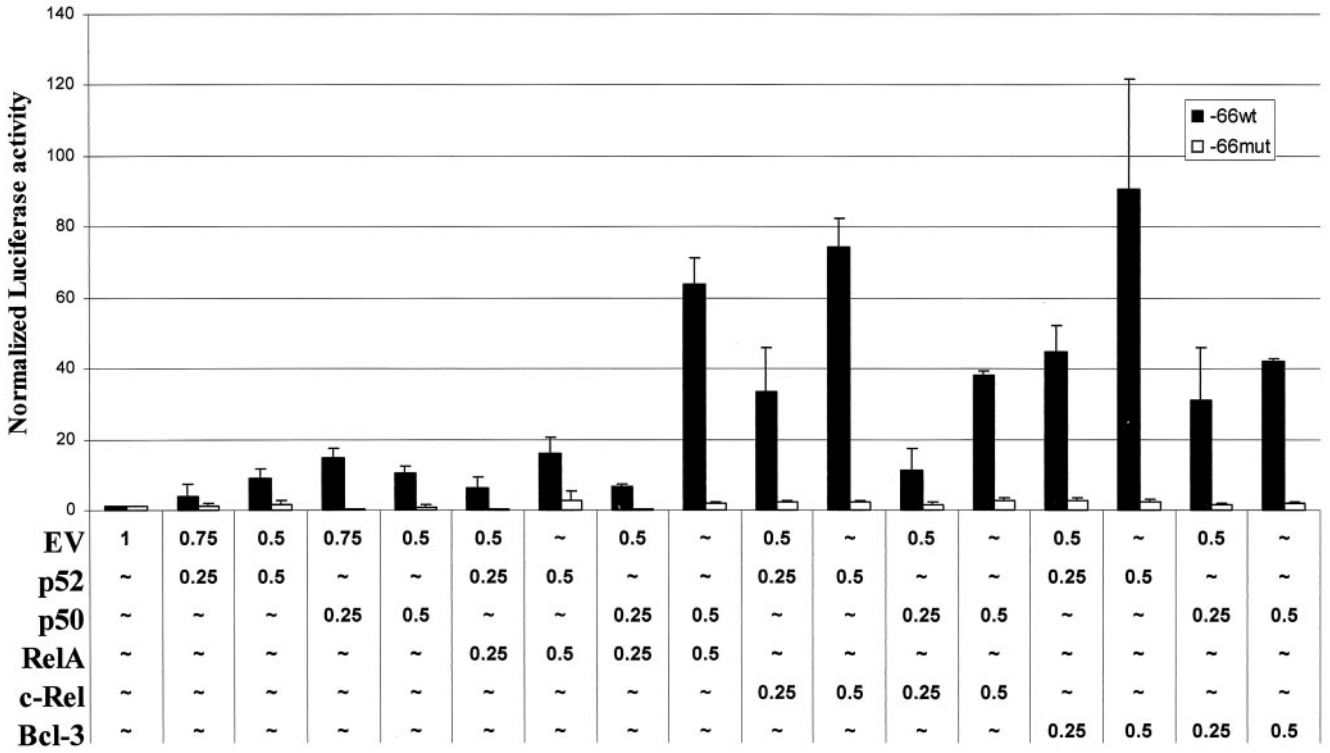

FIG. 11. c-Rel heterodimer complexes induce the cyclin D1 promoter. NMuMG cells were transfected, in duplicate, with −66 WT-cyclin D1 or −66 Mut-cyclin D1 luciferase gene reporter constructs and 0.5 μg of pSV40-β-gal in the presence of the indicated amounts of NF-κB or Bcl-3 plasmid expression vectors. After 48 h, cultures were harvested, normalized for β-Gal activity, and assayed for luciferase activity. Normalized values of luciferase activity are presented (error bars, standard deviations).

Ectopic c-Rel expression increases the levels of cyclin D1, p52, and p50 in mammary epithelial cells. Next, we investigated the effects of ectopic stable c-Rel overexpression on cyclin D1 and NF-κB subunit levels using MCF-10F human untransformed breast epithelial cells. Cells were transfected with a mouse c-Rel expression vector along with a puromycin selection plasmid, and grown in the presence of puromycin. Mixed populations of selected cells were used for analysis. As controls, we used either MCF-10F cells transfected with the pBluescript and puromycin selection plasmids and selected with puromycin or MCF-10F untransfected parental cells grown under the same conditions. (All populations of cells grew at the same rate, data not shown). We first confirmed overexpression of c-Rel in WCEs of c-Rel-transfected cells compared to parental MCF-10F cells, using β-actin as control for equal loading (Fig. 12). Scanning of this and a duplicate experiment indicated that c-Rel-transfected MCF-10F cells displayed a (2.5 ± 1.2)-fold increase in total level of c-Rel compared to control cells. Importantly, we observed that c-Rel-transfected MCF-10F cells displayed significant increases in the levels of cyclin D1, as well as in the NF-κB subunits p52 and p50 (and their precursors p100 and p105) compared to control cells (Fig. 12). Densitometry analysis indicated that c-Rel-transfected MCF-10F cells displayed a 3.1 and 2.7-fold increase in levels of p52 and p50, respectively. Densitometry of this and two other immunoblots (data not shown) indicated that c-Rel-transfected MCF-10F cells displayed a (1.8 ± 0.1)-fold increase in levels of cyclin D1. Essentially no change was observed in levels of RelA in c-Rel transfected cells. Control puromycin-selected cells displayed levels of c-Rel and cyclin D1 comparable to the parental untransfected cells MCF-10F cells, indicating that the selection was not responsible for this observed increase (data not shown). Thus, sustained overexpression of c-Rel in untransformed mammary epithelial cells leads to increased levels of p52, p50, and cyclin D1 expression. Overall, these findings indicate sustained c-Rel expression in mammary epithelial cells is sufficient to lead to the induction in NF-κB subunits and target genes, responsible for mammary tumorigenesis.

DISCUSSION

Here we demonstrate for the first time that c-Rel plays a causal role in tumorigenesis of the mammary gland in an MMTV-LTR-driven mouse model. Overall, one or more mammary tumors were detected in 31.6% of MMTV–c-*rel* transgenic mice at an average age of 19.9 months. Histological analysis of the mammary tumors in four independent lines provided evidence for a wide spectrum of tumor subtypes, including adenocarcinomas, adenosquamous carcinomas, squamous carcinomas, a spindle cell carcinoma and a papillary carcinoma. One mouse developed pulmonary metastasis in addition to multiple mammary adenocarcinomas. In addition to mammary carcinomas, several mice had enlarged spleen or other abnormalities including lymphoid or myeloid hyperplasia or centrocytic lymphomas in the spleen, which can be correlated to the expression of the transgene in

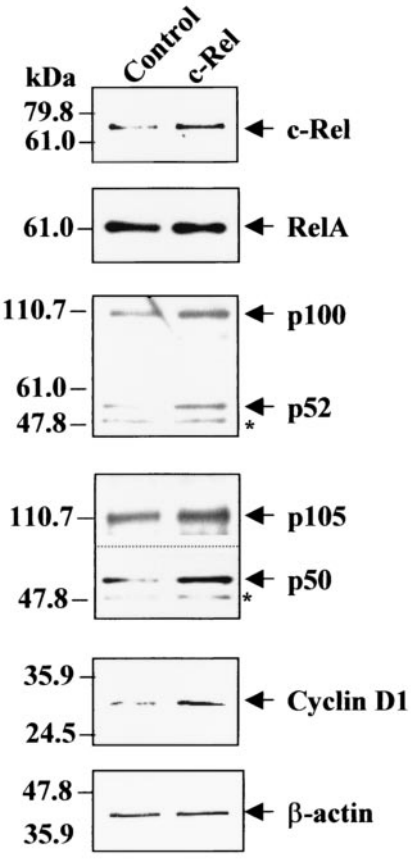

FIG. 12. Ectopic c-Rel expression in human MCF-10F untransformed mammary epithelial cells induces levels of cyclin D1, and p52 and p50 expression. Cells were cotransfected with pSVSport-c-Rel (c-Rel) and pGKpuro plasmid expression vectors, and a mixed population of cells was selected with puromycin. WCEs were prepared from transfected cells and parental untransfected cells (Control), and samples (10 μg) were subjected to immunoblot analysis for c-Rel, RelA, p52/p100, p50/p105, cyclin D1, and β-actin levels using two identical blots that were successively reprobed with the different antibodies. To better visualize the p105 versus p50 band, a longer exposure was used.

splenocytes (Fig. 1B), as also shown in previous studies of mammary tumors in transgenic mice using the same promoter (55, 67). By contrast, only rare (<1%) spontaneous cases of mammary tumors were reported in the FVB/N strain, consisting of squamous carcinomas or keratoacanthomas (42). Although the types of some tumors seen here are similar to those found in elderly FVB/N mice, the range and types of mammary tumors seen in the MMTV–c-*rel* mice are entirely similar to those induced by the *wnt* pathway (51). The MMTV–c-*rel* mammary tumors displayed sustained expression of the c-*rel* transgene mRNA. Tumors were also typified by overexpression of c-Rel protein, and displayed elevated mRNA levels of cyclin D1, c-*myc*, and *bcl-xl*, three NF-κB target genes implicated in control of growth and cell survival. These increases were detected in normal mammary glands during and after the first cycle of pregnancy. Stable ectopic c-Rel expression in untransformed mammary epithelial cells led to elevated levels of p50/p105, p52/p100, and cyclin D1. Furthermore, c-Rel complexes with p50 or p52 activated the cyclin D1 promoter. While the v-*rel* oncogene has been shown to be highly tumorigenic, our findings represent the first in vivo demonstration of the transforming ability of the c-Rel NF-κB subunit.

As expected, expression of c-*rel* transgene mRNA was detected in all the c-Rel-induced tumors tested and also in some grossly normal transgenic mammary glands from multiparous mice. Total c-Rel protein and c-*rel* transgene mRNA levels did not always correlate (Fig. 3 and 5), suggesting that endogenous c-Rel levels increased in some MMTV–c-*rel* mammary tumors, as observed previously in patient primary tumor samples (15, 59). Importantly, supershift analysis confirmed the presence of c-Rel in the NF-κB DNA binding complexes (Fig. 4 and data not shown). EMSA and immunoblot analysis demonstrated the induction of other NF-κB subunits in the MMTV–c-*rel* tumor mammary gland samples, including p50, p52, RelB, and RelA and the Bcl-3 protein in addition to the expected c-Rel. The vast majority of primary breast cancer specimens from patients display activation of multiple NF-κB proteins, including c-Rel, p50, p52, RelA, and RelB subunits and Bcl-3 protein (15, 59). The genes encoding the p105/p50, p100/p52 and RelB proteins are known NF-κB targets (reviewed in reference 44), and ectopic c-Rel expression in MCF-10F cells induced p50 and p52 expression. These findings suggest that direct activation by c-Rel may be responsible for the induction of these NF-κB subunits. In the case of Bcl-3, we have found that it is normally expressed in the mouse mammary epithelial cell during development (data not shown). A modest increase in the RelA subunit level was observed in MMTV–c-*rel* tumors by immunoblot, but not in the c-Rel transfected mammary epithelial cells. It is likely that the elevated level of this subunit observed in the tumors results indirectly from overexpression of the c-Rel transgene, e.g., activation of a cytokine such as tumor necrosis factor. If true, posttranslational modifications might explain the relatively high levels of RelA-containing complexes observed in EMSA in many of the mammary tumor samples. Lastly, it appeared that the level of c-Rel-containing complexes was relatively low, compared to the RelA subunit, suggesting that c-Rel may need to be modified or to interact with other proteins to efficiently bind the DNA. We are currently investigating factors that regulate c-Rel binding in mammary epithelial cells.

NF-κB appears to promote cell proliferation and survival of mammary epithelial cells in culture (34, 58, 59) and development of the normal mammary gland (8, 10, 14). Thus, we hypothesized that c-Rel activation could contribute to tumor cell growth and survival through the induction of a number of NF-κB target genes, including regulators of cell cycle, proliferation, and survival (reviewed in references 33 and 47). The cyclin D1 promoter, which contains several κB elements, can be activated by classical NF-κB (25, 26), although a more recent study has found Bcl-3/p52-mediated activation of the cyclin D1 promoter more potent (65). Here, we demonstrate the ability of c-Rel/p52 and c-Rel/p50 complexes to induce the cyclin D1 promoter. Female mice null for the cyclin D1 gene fail to develop normal mammary glands (18, 54). Overexpression of cyclin D1 has been implicated in breast cancer in humans and in rodent models (reviewed in reference 32). Importantly, MMTV-cyclin D1 mice develop mammary cancers (64), and an increase in levels of cyclin D1 was seen in mammary tumors that develop in mice with enforced expression of Neu or Ras (68). Female mice deficient for cyclin D1 are

totally resistant to breast cancers induced by Neu and Ras, but not to those induced by Myc and Wnt-1 (68). Interestingly, several groups, including our own, have shown that activated Neu and Ras induce functional NF-κB in mammary and liver epithelial and fibroblast cells (4, 19, 45, 69). The human and mouse c-*myc* promoters have been shown to be targets of NF-κB complexes, including those containing c-Rel, in breast epithelial and other cell types (17, 31, 34, 39, 40). The c-*myc* gene is overexpressed in breast neoplasia samples from patients (reviewed in reference 41). Targeted c-*myc* expression in the mammary gland of mice using either the MMTV-LTR or the whey acidic protein promoter leads to mammary tumor development (53, 61). The promoter of the *bcl-xl* cell death antagonist gene is regulated by p50-p65- or p50–c-Rel-containing complexes (13, 70). In the present study, we observed that many of the MMTV–c-*rel* tumors display elevated levels of expression of cyclin D1, *bcl-xl*, and c-*myc* RNA. No correlation was noted between tumor latency and cyclin D1 levels; while, some correlation was seen with c-*myc* mRNA levels, although, tumors numbers were too low to draw any firm conclusions. Change in expression profiles of these transcripts were also observed in normal mammary glands of transgenic mice, starting the first cycle of gestation. Transfection analysis suggested that the genes encoding cyclin D1, p52 and p50 are c-Rel targets. These findings are consistent with a direct role of c-Rel in the events leading to mammary gland tumorigenesis in the MMTV–c-*rel* mice.

Aberrant activation of nuclear NF-κB/Rel has been found to correlate with oncogenesis in several other systems, including thyroid carcinoma, non-small cell lung carcinoma, colon carcinoma, ovarian carcinoma, prostate cancer (62), Hodgkin's disease (6), and various types of lymphomas (reviewed in reference 47). In hematopoietic tumors, amplification, overexpression, or rearrangement of the c-*rel*, *nf-kb1*, *nf-kb2*, or *relA* genes or the *bcl-3* gene; and mutations inactivating the IκB-α protein have been noted (reviewed in reference 47). Therefore, increased activation of NF-κB can occur by multiple mechanisms in tumor cells. Similarly, in breast cancer cells, multiple mechanisms that lead to aberrant activation of NF-κB have also been described. We observed that many human breast tumor specimens and cell lines in culture display constitutive IKK and protein kinase CK2 activity, which correlated with elevated levels of NF-κB binding activity (50). Products of several oncogenes induce NF-κB activity in mammary epithelial cells, such as Her-2/neu (45, 69). Presumably these oncogenes require downstream effectors that activate either an IκB kinase or CK2, thereby increasing the rate of IκB turnover, and basal NF-κB nuclear translocation and binding to the DNA. Recently, we have demonstrated that activation of NF-κB by Her-2 can be reduced upon inhibition of CK2 (49). Interestingly, in vitro studies have suggested that Her-2/neu, Ras, and Raf preferentially induce classical p50/RelA complexes (4, 19, 45, 69). The late-onset of tumor development and the variety in the tumor histological patterns in MMTV–c-*rel* mice implies the requirement of additional pathways in the mammary gland tumorigenesis, in addition to overexpression of c-Rel. This phenotype is very reminiscent of the MMTV-CK2α subunit transgenic mice, which we have described recently (38). These mice developed late-onset mammary carcinomas of heterogeneous histological profiles. Consistent with the biological properties of CK2, the MMTV-CK2 tumors expressed high levels of NF-κB activity, c-Myc, and activated β-catenin (38). Altogether, our findings indicate that cellular c-Rel is able to transform mammal epithelial cells in vivo and thus represents a potential therapeutic target.

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R.R.-M. and D.W.K. contributed equally to this work.

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REFERENCES

1. **Abbadie, C., N. Kabrun, F. Bouali, J. Smardova, D. Stehelin, B. Vandenbunder, and P. J. Enrietto.** 1993. High levels of c-rel expression are associated with programmed cell death in the developing avian embryo and in bone marrow cells in vitro. Cell **75:**899–912.
2. **Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, Jr., L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, L. M. Staudt, and et al.** 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature **403:**503–511.
3. **Arsura, M., M. J. FitzGerald, N. Fausto, and G. E. Sonenshein.** 1997. Nuclear factor-κB/Rel blocks transforming growth factor beta1-induced apoptosis of murine hepatocyte cell lines. Cell Growth Differ. **8:**1049–1059.
4. **Arsura, M., F. Mercurio, A. L. Oliver, S. S. Thorgeirsson, and G. E. Sonenshein.** 2000. Role of the IκB kinase complex in oncogenic Ras- and Raf-mediated transformation of rat liver epithelial cells. Mol. Cell. Biol. **20:**5381–5391.
5. **Arsura, M., and G. E. Sonenshein.** 2001. The roles of the c-myc and c-myb oncogenes in hematopoiesis and leukemogenesis, p. 521–549. *In* K. Ravid and J. Licht (ed.), Transcription factors: normal and malignant development of blood cells. Wiley-Liss Inc., New York, N.Y.
6. **Bargou, R. C., C. Leng, D. Krappmann, F. Emmerich, M. Y. Mapara, K. Bommert, H. D. Royer, C. Scheidereit, and B. Dorken.** 1996. High-level nuclear NF-kappa B and Oct-2 is a common feature of cultured Hodgkin/Reed-Sternberg cells. Blood **87:**4340–4347.
7. **Bours, V., G. Franzoso, V. Azarenko, S. Park, T. Kanno, K. Brown, and U. Siebenlist.** 1993. The oncoprotein Bcl-3 directly transactivates through kappa B motifs via association with DNA-binding p50B homodimers. Cell **72:**729–739.
8. **Brantley, D. M., C. L. Chen, R. S. Muraoka, P. B. Bushdid, J. L. Bradberry, F. Kittrell, D. Medina, L. M. Matrisian, L. D. Kerr, and F. E. Yull.** 2001. Nuclear factor-κB (NF-κB) regulates proliferation and branching in mouse mammary epithelium. Mol. Biol. Cell **12:**1445–1455.
9. **Calaf, G., and J. Russo.** 1996. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis **14:**483–492.
10. **Cao, Y., G. Bonizzi, T. N. Seagroves, F. R. Greten, R. Johnson, E. V. Schmidt, and M. Karin.** 2001. IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell **107:**763–775.
11. **Cardiff, R. D., and R. J. Munn.** 1995. Comparative pathology of mammary tumorigenesis in transgenic mice. Cancer Lett. **90:**13–19.
12. **Catz, S. D., and J. L. Johnson.** 2001. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. Oncogene **20:**7342–7351.
13. **Chen, C., L. C. Edelstein, and C. Gelinas.** 2000. The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-x_L. Mol. Cell. Biol. **20:**2687–2695.
14. **Clarkson, R. W., J. L. Heeley, R. Chapman, F. Aillet, R. T. Hay, A. Wyllie, and C. J. Watson.** 2000. NF-κB inhibits apoptosis in murine mammary epithelia. J. Biol. Chem. **275:**12737–12742.
15. **Cogswell, P. C., D. C. Guttridge, W. K. Funkhouser, and A. S. Baldwin, Jr.** 2000. Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. Oncogene **19:**1123–1131.
16. **Dean, M., R. B. Kent, and G. E. Sonenshein.** 1983. Transcriptional activation of immunoglobulin alpha heavy-chain genes by translocation of the c-myc oncogene. Nature **305:**443–446.

17. **Duyao, M. P., A. J. Buckler, and G. E. Sonenshein.** 1990. Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter. Proc. Natl. Acad. Sci. USA **87:**4727–4731.
18. **Fantl, V., G. Stamp, A. Andrews, I. Rosewell, and C. Dickson.** 1995. Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev. **9:**2364–2372.
19. **Finco, T. S., J. K. Westwick, J. L. Norris, A. A. Beg, C. J. Der, and A. S. Baldwin, Jr.** 1997. Oncogenic Ha-Ras-induced signaling activates NF-κB transcriptional activity, which is required for cellular transformation. J. Biol. Chem. **272:**24113–24116.
20. **Fujita, T., G. P. Nolan, H. C. Liou, M. L. Scott, and D. Baltimore.** 1993. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev. **7:**1354–1363.
21. **Ghosh, S., and M. Karin.** 2002. Missing pieces in the NF-κB puzzle. Cell **109:**S81–96.
22. **Gilmore, T. D.** 1999. Multiple mutations contribute to the oncogenicity of the retroviral oncoprotein v-Rel. Oncogene **18:**6925–6937.
23. **Gilmore, T. D., C. Cormier, J. Jean-Jacques, and M. E. Gapuzan.** 2001. Malignant transformation of primary chicken spleen cells by human transcription factor c-Rel. Oncogene **20:**7098–7103.
24. **Grumont, R. J., A. Strasser, and S. Gerondakis.** 2002. B cell growth is controlled by phosphatidylinosotol 3-kinase-dependent induction of Rel/NF-κB regulated c-myc transcription. Mol. Cell **10:**1283–1294.
25. **Guttridge, D. C., C. Albanese, J. Y. Reuther, R. G. Pestell, and A. S. Baldwin, Jr.** 1999. NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol. Cell. Biol. **19:**5785–5799.
26. **Hinz, M., D. Krappmann, A. Eichten, A. Heder, C. Scheidereit, and M. Strauss.** 1999. NF-κB function in growth control: regulation of cyclin D1 expression and G_0/G_1-to-S-phase transition. Mol. Cell. Biol. **19:**2690–2698.
27. **Hrdlickova, R., J. Nehyba, and E. H. Humphries.** 1994. In vivo evolution of c-rel oncogenic potential. J. Virol. **68:**2371–2382.
28. **Huang, D. B., Y. Q. Chen, M. Ruetsche, C. B. Phelps, and G. Ghosh.** 2001. X-ray crystal structure of proto-oncogene product c-Rel bound to the CD28 response element of IL-2. Structure (Cambridge) **9:**669–678.
29. **Jager, R., U. Herzer, J. Schenkel, and H. Weiher.** 1997. Overexpression of Bcl-2 inhibits alveolar cell apoptosis during involution and accelerates c-myc-induced tumorigenesis of the mammary gland in transgenic mice. Oncogene **15:**1787–1795.
30. **Jeay, S., G. E. Sonenshein, M. C. Postel-Vinay, and E. Baixeras.** 2000. Growth hormone prevents apoptosis through activation of nuclear factor-κB in interleukin-3-dependent Ba/F3 cell line. Mol. Endocrinol. **14:**650–661.
31. **Ji, L., M. Arcinas, and L. M. Boxer.** 1994. NF-κ B sites function as positive regulators of expression of the translocated c-*myc* allele in Burkitt's lymphoma. Mol. Cell. Biol. **14:**7967–7974.
32. **Joyce, D., C. Albanese, J. Steer, M. Fu, B. Bouzahzah, and R. G. Pestell.** 2001. NF-κB and cell-cycle regulation: the cyclin connection. Cytokine Growth Factor Rev. **12:**73–90.
33. **Karin, M., Y. Cao, F. R. Greten, and Z. W. Li.** 2002. NF-κB in cancer: from innocent bystander to major culprit. Nat. Rev. Cancer **2:**301–310.
34. **Kim, D. W., M. A. Sovak, G. Zanieski, G. Nonet, R. Romieu-Mourez, A. W. Lau, L. J. Hafer, P. Yaswen, M. Stampfer, A. E. Rogers, J. Russo, and G. E. Sonenshein.** 2000. Activation of NF-κB/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis **21:**871–879.
35. **Kindy, M. S., and G. E. Sonenshein.** 1986. Regulation of oncogene expression in cultured aortic smooth muscle cells. Post-transcriptional control of c-myc mRNA. J. Biol. Chem. **261:**12865–12868.
36. **Kontgen, F., R. J. Grumont, A. Strasser, D. Metcalf, R. Li, D. Tarlinton, and S. Gerondakis.** 1995. Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. Genes Dev. **9:**1965–1977.
37. **Kurland, J. F., R. Kodym, M. D. Story, K. B. Spurgers, T. J. McDonnell, and R. E. Meyn.** 2001. NF-κB1 (p50) homodimers contribute to transcription of the bcl-2 oncogene. J. Biol. Chem. **276:**45380–45386.
38. **Landesman-Bollag, E., R. Romieu-Mourez, D. H. Song, G. E. Sonenshein, R. D. Cardiff, and D. C. Seldin.** 2001. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene **20:**3247–3257.
39. **La Rosa, F. A., J. W. Pierce, and G. E. Sonenshein.** 1994. Differential regulation of the c-*myc* oncogene promoter by the NF-κB *rel* family of transcription factors. Mol. Cell. Biol. **14:**1039–1044.
40. **Lee, H., M. Arsura, M. Wu, M. Duyao, A. J. Buckler, and G. E. Sonenshein.** 1995. Role of Rel-related factors in control of c-myc gene transcription in receptor-mediated apoptosis of the murine B cell WEHI 231 line. J. Exp. Med. **181:**1169–1177.
41. **Liao, D. J., and R. B. Dickson.** 2000. c-Myc in breast cancer. Endocr. Relat. Cancer **7:**143–164.
42. **Mahler, J. F., W. Stokes, P. C. Mann, M. Takaoka, and R. R. Maronpot.** 1996. Spontaneous lesions in aging FVB/N mice. Toxicol. Pathol. **24:**710–716.
43. **Nakshatri, H., P. Bhat-Nakshatri, D. A. Martin, R. J. Goulet, Jr., and G. W. Sledge, Jr.** 1997. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol. **17:**3629–3639.
44. **Pahl, H. L.** 1999. Activators and target genes of Rel/NF-κB transcription factors. Oncogene **18:**6853–6866.
45. **Pianetti, S., M. Arsura, R. Romieu-Mourez, R. J. Coffey, and G. E. Sonenshein.** 2001. Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IκB-alpha that can be inhibited by the tumor suppressor PTEN. Oncogene **20:**1287–1299.
46. **Ravid, K., D. L. Beeler, M. S. Rabin, H. E. Ruley, and R. D. Rosenberg.** 1991. Selective targeting of gene products with the megakaryocyte platelet factor 4 promoter. Proc. Natl. Acad. Sci. USA **88:**1521–1525.
47. **Rayet, B., and C. Gelinas.** 1999. Aberrant rel/nfkb genes and activity in human cancer. Oncogene **18:**6938–6947.
48. **Rice, N. R., and M. K. Ernst.** 1993. In vivo control of NF-kappa B activation by I kappa B alpha. EMBO J. **12:**4685–4695.
49. **Romieu-Mourez, R., E. Landesman-Bollag, D. C. Seldin, and G. E. Sonenshein.** 2002. Protein kinase CK2 promotes aberrant activation of NF-κB, transformed phenotype and survival of breast cancer cells. Cancer Res. **62:**6770–6778.
50. **Romieu-Mourez, R., E. Landesman-Bollag, D. C. Seldin, A. M. Traish, F. Mercurio, and G. E. Sonenshein.** 2001. Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-κB in breast cancer. Cancer Res. **61:**3810–3818.
51. **Rosner, A., K. Miyoshi, E. Landesman-Bollag, X. Xu, D. C. Seldin, A. R. Moser, C. L. MacLeod, G. Shyamala, A. E. Gillgrass, and R. D. Cardiff.** 2002. Pathway pathology: histological differences between ErbB/Ras and Wnt pathway transgenic mammary tumors. Am. J. Pathol. **161:**1087–1097.
52. **Schneider, T. J., D. Grillot, L. C. Foote, G. E. Nunez, and T. L. Rothstein.** 1997. Bcl-x protects primary B cells against Fas-mediated apoptosis. J. Immunol. **159:**4834–4839.
53. **Schoenenberger, C. A., A. C. Andres, B. Groner, M. van der Valk, M. LeMeur, and P. Gerlinger.** 1988. Targeted c-myc gene expression in mammary glands of transgenic mice induces mammary tumours with constitutive milk protein gene transcription. EMBO J. **7:**169–175.
54. **Sicinski, P., J. L. Donaher, S. B. Parker, T. Li, A. Fazeli, H. Gardner, S. Z. Haslam, R. T. Bronson, S. J. Elledge, and R. A. Weinberg.** 1995. Cyclin D1 provides a link between development and oncogenesis in the retina and breast. Cell **82:**621–630.
55. **Sinn, E., W. Muller, P. Pattengale, I. Tepler, R. Wallace, and P. Leder.** 1987. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell **49:**465–475.
56. **Sonenshein, G. E.** 1997. Rel/NF-kappa B transcription factors and the control of apoptosis. Semin. Cancer Biol. **8:**113–119.
57. **Song, D. H., D. J. Sussman, and D. C. Seldin.** 2000. Endogenous protein kinase CK2 participates in Wnt signaling in mammary epithelial cells. J. Biol. Chem. **275:**23790–23797.
58. **Sovak, M. A., M. Arsura, G. Zanieski, K. T. Kavanagh, and G. E. Sonenshein.** 1999. The inhibitory effects of transforming growth factor beta1 on breast cancer cell proliferation are mediated through regulation of aberrant nuclear factor-κB/Rel expression. Cell Growth Differ. **10:**537–544.
59. **Sovak, M. A., R. E. Bellas, D. W. Kim, G. J. Zanieski, A. E. Rogers, A. M. Traish, and G. E. Sonenshein.** 1997. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Investig. **100:**2952–2960.
60. **Stanton, L. W., R. Watt, and K. B. Marcu.** 1983. Translocation, breakage and truncated transcripts of c-myc oncogene in murine plasmacytomas. Nature **303:**401–406.
61. **Stewart, T. A., P. K. Pattengale, and P. Leder.** 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell **38:**627–637.
62. **Sumitomo, M., M. Tachibana, J. Nakashima, M. Murai, A. Miyajima, F. Kimura, M. Hayakawa, and H. Nakamura.** 1999. An essential role for nuclear factor kappa B in preventing TNF-alpha-induced cell death in prostate cancer cells. J. Urol. **161:**674–679.
63. **Thiery, J. P.** 2002. Epithelial-mesenchymal transitions in tumour progression. Nat. Rev. Cancer **2:**442–454.
64. **Wang, T. C., R. D. Cardiff, L. Zukerberg, E. Lees, A. Arnold, and E. V. Schmidt.** 1994. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature **369:**669–671.
65. **Westerheide, S. D., M. W. Mayo, V. Anest, J. L. Hanson, and A. S. Baldwin, Jr.** 2001. The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G_1 transition. Mol. Cell. Biol. **21:**8428–8436.
66. **Wu, M., H. Lee, R. E. Bellas, S. L. Schauer, M. Arsura, D. Katz, M. J. FitzGerald, T. L. Rothstein, D. H. Sherr, and G. E. Sonenshein.** 1996. Inhibition of NF-κB/Rel induces apoptosis of murine B cells. EMBO J. **15:**4682–4690.
67. **Yang, Y. A., O. Dukhanina, B. Tang, M. Mamura, J. J. Letterio, J. MacGregor, S. C. Patel, S. Khozin, Z. Y. Liu, J. Green, M. R. Anver, G. Merlino, and L. M. Wakefield.** 2002. Lifetime exposure to a soluble TGF-beta antagonist protects mice against metastasis without adverse side effects. J. Clin. Investig. **109:**1607–1615.
68. **Yu, Q., Y. Geng, and P. Sicinski.** 2001. Specific protection against breast cancers by cyclin D1 ablation. Nature **411:**1017–1021.
69. **Zhou, B. P., M. C. Hu, S. A. Miller, Z. Yu, W. Xia, S. Y. Lin, and M. C. Hung.** 2000. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-κB pathway. J. Biol. Chem. **275:**8027–8031.
70. **Zong, W. X., L. C. Edelstein, C. Chen, J. Bash, and C. Gelinas.** 1999. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNFα-induced apoptosis. Genes Dev. **13:**382–387.

IKK-i/IKKε Expression is Induced by CK2 and Promotes Aberrant NF-κB Activation in Breast Cancer Cells

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Abstract

Aberrant activation of NF-κB transcription factors has been implicated in the pathogenesis of breast cancer. We previously demonstrated elevated activity of IKKα, IKKβ and protein kinase CK2 in primary human breast cancer specimens and in cultured cells. A novel inducible IKK protein termed IKK-i/IKKε has been characterized as a potential NF-κB activator. Here we provide evidence that implicates IKK-i/IKKε in the pathogenesis of breast cancer. We demonstrate IKK-i/IKKε expression in primary human breast cancer specimens and carcinogen-induced mouse mammary tumors. Multiple breast cancer cell lines showed higher levels of IKK-i/IKKε and kinase activity compared to untransformed MCF-10F breast epithelial cells. Interestingly, IKK-i/IKKε expression correlated with CK2α expression in mammary glands and breast tumors derived from MMTV-CK2α transgenic mice. Ectopic CK2 expression in untransformed cells led to increased IKK-i/IKKε mRNA and protein levels. Inhibition of CK2α via the pharmacological inhibitor apigenin or upon transfection of a CK2 kinase inactive subunit reduced IKK-i/IKKε levels. Expression of a kinase inactive IKK-i/IKKε mutant in breast cancer cells reduced NF-κB activity as judged by transfection assays of reporters driven either by NF-κB elements or the promoters of two NF-κB target genes, *cyclinD1* and *relB*. Importantly, the kinase inactive IKK-i/IKKε mutant reduced the endogenous levels of these genes as well as the ability of breast cancer cells to grow in soft agar or form invasive colonies in matrigel. Thus, CK2 induces functional IKK-i/IKKε, which is an important mediator of the activation of NF-κB that plays a critical role in the pathogenesis of breast cancer.

Introduction

Nuclear Factor (NF)-κB/Rel is a family of dimeric transcription factors distinguished by the presence of a 300 amino acid region, termed the Rel homology domain, which determines much of its function (1). Classical NF-κB is a heterodimer composed of a RelA (p65) and a p50 subunit. In most cells, NF-κB/Rel proteins are sequestered in the cytoplasm bound to the specific IκB inhibitory proteins, of which IκB-α is the paradigm. While the v-*rel* gene, carried by the highly oncogenic avian reticuloendotheliosis virus strain T (Rev-T) is able to cause tumors in birds, the role of NF-κB in mammalian cancers was less clear for many years (2), although several oncogenic mammalian viruses were shown to activate NF-κB. For example, the product of the *tax* gene of the HTLV-1 virus activates NF-κB (3), which we showed mediates transactivation of the c-*myc* promoter (4, 5). Recently, we and others have demonstrated a role for NF-κB/Rel factors in breast cancer (6, 7). High levels of nuclear NF-κB/Rel were found in human breast tumor cell lines, carcinogen-transformed mammary epithelial cells, and the majority of primary human or rodent breast tumor tissue samples. In contrast, untransformed breast epithelial cells and normal rat mammary glands contained low basal levels (6, 7).

The increased NF-κB/Rel activity in tumor cells has been correlated with a decrease in stability of IκB proteins, in particular of IκB-α, which permits the released NF-κB subunits to translocate into the nucleus (8). To begin to elucidate the mechanism of this increased turnover, we recently characterized the activity of several kinases implicated in IκB-α turnover. Breast cancer specimens and tumor cells displayed higher levels of activity of either the IκB kinase IKKα or IKKβ proteins or of the serine/threonine protein kinase CK2 (9, 10). Phosphorylation of IκB-α at two serine residues (Ser32 and Ser36) by IKKα or IKKβ, which are present in a large IKK complex containing multiple copies of a regulatory subunit NEMO/IKKγ (11, 12), leads to

IκB-α ubiquination and subsequent proteasome-mediated degradation in the canonical NF-κB induction pathway. In addition to the N-terminal phosphorylation of IκB-α, it has been shown that C-terminal phosphorylation via CK2 within the C-terminal PEST [Ser-283, Ser-289, T-291 and Ser-293] domain of IκB-α also affects its stability (13, 14). CK2 is a ubiquitously expressed tetrameric protein kinase containing two catalytic (α/α, α'/α or α'/α') and two regulatory (β/β) subunits (15, 16). Recent evidence suggests that CK2 activity can be altered by cellular stress, including UV irradiation (17, 18). CK2-mediated phosphorylation of IκB-α has been implicated in basal and signal-independent turnover of IκB-α (13, 19-21). These findings have implicated CK2 in control of intrinsic IκB-α stability and thus activation of NF-κB. Importantly, we showed that CK2 levels are elevated in primary human breast cancer specimens as well as in established cell lines with elevated NF-κB activity (9, 10, 22, 23).

A recently discovered inducible IKK protein also leads to IκB-α phosphorylation and NF-κB activation. Initially isolated from mice and termed IKK-i, it is also known as IKKε in humans (24, 25). IKK-i/IKKε is part of an independent complex containing TANK and TRAF (26). Tank Binding Kinase, TBK-1, which is highly homologous to IKK-i/IKKε, binds to TANK and TRAF and may form an alternative IKK complex consisting of IKK-i/IKKε and TBK-1 (27). IKK-i/IKKε appears to specifically phosphorylate IκB-α at Ser36 (24, 25). While the significance of only one phosphorylation event at the N-terminus of IκB-α is not yet entirely clear, it may pre-dispose IκB-α towards Ser32 phosphorylation and subsequent degradation (25). Furthermore, IKK-i/IKKε directly phosphorylates transcription factors (26), including NF-κB/Rel factors (28, 29). Given the aberrant activation of NF-κB in malignancies, we investigated the role of IKK-i/IKKε in breast cancer. Here, we demonstrate, for the first time, IKK-i/IKKε

induction in primary human breast cancers, rodent mammary tumors and cell lines in culture. Furthermore, we implicate CK2 in the elevated IKK-i/IKKε levels and show a link between IKK-i/IKKε and activation of NF-κB in breast cancer.

MATERIALS AND METHODS

Human breast cancer specimen analysis. Primary human breast cancer tissue specimens were obtained from patients undergoing surgery for treatment of breast cancer with approval of the Institutional Review Board of Boston Medical Center and have previously been described (30).

Transgenic mice. Creation of the transgenic MMTV-c-*rel* and MMTV-CK2 transgenic was previously described (22, 30). Breeding of MMTV-CK2 mice and MMTV-c-*rel* mice created bi-transgenic MMTV-CK2 X c-*rel* mice. Mice were housed in a 2-way barrier at the Boston University School of Medicine Transgenic mouse facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care. Wild type mice used in the study were age- and pregnancy-matched. All tissues were frozen immediately after extraction in liquid nitrogen and stored at –80°C.

Carcinogen treatment of mice. Twenty virgin female FVB/N mice, housed in a 2-way barrier, were treated according to a protocol approved by the Boston University Institutional Animal Care and Use Committee. Mice were each given six weekly 1.0 mg doses of 7,12-dimethylbenz(*a*)anthracene (DMBA) in 0.2 ml of sesame oil by oral gavage, beginning at 5 weeks of age. Mice were then mated continuously to provide an oscillating hormonal environment and followed until either tumors developed or the mice died. By 34 weeks of age (29 weeks after beginning DMBA treatment), all mice had developed tumors. Mice bearing

tumors >0.5 cm were euthanized by CO_2 inhalation and necropsied. Mammary tumors and grossly normal mammary glands from parous age-matched control FVB mice were excised and frozen on dry ice and stored at -80^0C. Whole cell protein extracts were prepared by homogenizing frozen tumors or mammary gland specimens in lysis buffer containing a cocktail of protease inhibitors (50mM Tris-HCl pH 8.0, 1% Nonidet P-40, 125mM NaCl, 1mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM Na3VO4, and 10 mM sodium pyrophosphate).

Cell culture and treatment conditions. Hs578T, and MDA-MB-231 breast cancer cell lines were grown in standard culture medium, as described by the American Tissue Culture Collection. MCF-10F and D3-1, a DMBA-transformed MCF-10F derivative (31), were cultured as described (9). The MMTV-Her2/neu NF639 cell line was derived from mammary tumors expressing Her2/neu and cultured as previously described (32). Human HEK293T endothelial derived kidney cells were cultured in DMEM supplemented with 10% FBS (Invitrogen). NIH 3T3 fibroblast cell lines were cultured as described (10). Where indicated, cells were incubated with 25 or 50 μM apigenin (Sigma) dissolved in DMSO or treated with vehicle DMSO alone.

Plasmids and transfection analyses. The pCDNA3-FLAG-IKKε and pCDNA3-FLAG-IKKε K38A vectors expressing IKKε and kinase inactive IKKε, respectively, were a kind gift of T. Maniatis (Harvard University, Cambridge MA) (24). The pRC/CMV-HA-CK2α', pRC/CMV-HA CK2α K68A and pRC/CMV-myc-CK2β vectors were provided by D. Litchfield (University of Western Ontario, Canada (33)). To evaluate NF-κB activity, 2-copy wild type or mutant NF-κB element-thymidine kinase promoter-chloramphenical acetyl transferase (CAT) reporter vectors (E8-CAT and mut-E8-CAT, respectively) (5) or a 6-copy NF-κB-element driven luciferase reporter construct, kindly provided by G. Rawadi (Hoechst-Marion-Roussel,

Romainville, France) (34) were used. NF-κB-driven promoter constructs used include: 1) *cyclin D1* promoter: containing WT (-66 wt-Luc) or mutant (-66 mut-Luc) NF-κB elements (kind gift of R.G. Pestell, Lombardi Comprehensive Cancer Center, Washington DC) (30, 35); 2) *relB* promoter: containing WT (*p1.7 relB*) or mutant (*p1.7 mut-relB*) versions of the two NF-κB elements, prepared as described previously (36). For transfection into six well or P100 plates, 4 μg or 10 μg total DNA, respectively were transfected per sample. For transient transfection into Hs578T and MDA-MB 231 cells, cells were incubated for 16-24 hours with DNA and GenePorter2 (Gene Therapy Systems). Transfections into MCF-10F, D3-1 and NF639 cell lines, were performed by incubating for 16 hours in the presence of DNA and Fugene 6 Transfection Reagent (Roche). The calcium phosphate method of transfection (37) was used with HEK293T cells. CAT and luciferase assays were performed as described in (6) and (30), respectively. Co-transfection of an SV40-β-galactosidase (SV40-β-gal) expression vector was used to normalize for transfection efficiency, as described (9). Where indicated, standard deviation (S.D.) was calculated and significance determined using the Student t-test (*p<0.05, **p<0.01, ***p<0.005).

Immunoblotting. Cytoplasmic protein extracts were prepared in RSB buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40) or TKM buffer (10 mM Tris [pH 7.6], 10 mM KCl, 5 mM $MgCl_2$, 0.2% NP-40), where indicated. Nuclear extracts were prepared in DR buffer (20 mM HEPES [pH 7.9], 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol) or RIPA buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 5 mM EDTA and 1% sodium sarcosyl). Whole cell extracts (WCEs) were prepared in RIPA buffer or in kinase lysis buffer (20 mM Tris-HCl [7.6], 150 mM NaCl). Protease and phosphatase inhibitors were added to each protein preparation (10 mM NaF, 25 mM β-glycerophosphate, p-

nitrophenyl phosphate, 1 mM Na_3VO_4, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin). Protein concentration was determined by Lowry assay using the Bio-Rad reagent (Bio-Rad). WCEs for reporter assays were prepared in 1X reporter lysis buffer (Promega).

Antibodies IKK-i/IKKε antibodies (sc-9913, sc-5694), IκB-α (sc-203), IκB-β (sc-945), and HA (sc-805) were purchased from Santa Cruz. Antibodies against IκB-ε and c-Myc were a gift from N. Rice and M. Ernst (NCI, Fredrick MD) and S. Hann (Vanderbilt University, Nashville TN), respectively. Competition assays for IKK-i/IKKε were performed using 2 μg cognate peptide (sc-9913 P). Antibodies against cyclin D1 (06-137) were purchased from Upstate Biotechnology. FLAG (F-4042) and β-actin (AC-15) antibodies were purchased from Sigma.

IKK-i/IKKε kinase assay. Protein extracts and immunoprecipitations were performed as previously described (9, 10). Briefly, 300 μg (in 500 μl kinase lysis buffer) WCEs were precleared with protein A/G agarose Plus beads (Santa Cruz) and bovine anti-goat HRP secondary antibody (Santa Cruz) for 1 hour at 4°C prior to the addition of 2 μg of IKK-i/IKKε antibody (sc-9913). WCEs were split into three aliquots. One third was used for a kinase assay with GST-IκB-α as a substrate, one third for a kinase assay with GST-2N-IκB-α containing S32A and S36A mutations. All kinase assays were performed as described (9). The remaining one-third of immunoprecipitated proteins were subject to immunoblot analysis for IKK-i/IKKε using an antibody directed to a different epitope (sc-5694, K-14).

RT-PCR. RNA was isolated using Trizol (Invitrogen) reagent according to the manufacturer's protocol and was quantified by measuring A_{260}. The A_{260}:A_{280} ratios were between 1.8 and 2. RNA samples were prepared as described (30). PCR were performed in a

Thermal Cycler (MJ Research) for 32 cycles under these conditions: 94°C for 60 sec, 57°C for 45 sec and 72°C for 55 sec. Primer pairs for PCR were as follows: *ikk-i/ikke* (Forward, nucleic acid position 504) 5'-CGGAAGCTGAACCACCAGAA-3' and (Reverse, 976) 5'-CCAGTGGCTGCATGGTACAA-3'; *hmgb1* (Forward, 725) 5'-AGGAGGATGAAGAGGAATGAG-3' and (Reverse, 1025) 5'-GACTGTACCAGGCAAGGTTA-3'; *β-actin* (Forward, 516) 5'-CACTGGCATCGTGATGGACT-3' and (Reverse, 923) 5'-CGGATGTCCACGTCACACTT-3'.

Soft agar transformation assay. Stable Hs578T breast cancer cells were plated at 5 x 10^3 cells/ml in 0.35% top agarose (SeaPlaque Agarose, FMC Bioproducts, Rockland, ME) with a base agarose of 0.7% supplemented with complete medium and 1 mg/ml G418 (Sigma). Plates were subsequently incubated for 16 days in humidified incubator at 37°C. Cells were stained with 0.5 ml of 0.0005% crystal violet, and colonies were counted visually under 8X magnification.

Matrigel invasion assay. Matrigel (BD Biosciences) was diluted to a working concentration of 6.3 mg/ml. For matrigel assays, 200 µl of Matrigel was added into a 24-well tissue culture plate and incubated at 37^{o}C for 30 minutes. A single cell suspension of NF639 cells (5 x 10^5 cells/ml) in serum free medium (DMEM) was made by passing the cell suspension 5 times through a 21.5 gauge needle. Ten µl (5000 cells) was mixed with 190 µl of Matrigel and plated onto the solidified bottom layer. Complete medium was added and the plate incubated at 37^{o}C for 3 to 6 days and photographed.

RESULTS

IKK-i/IKKε is expressed in primary human breast cancer specimens. To begin to assess the role of IKK-i/IKKε in breast cancer, cytoplasmic extracts from six human breast cancer tissue specimens were analyzed for expression of IKK-i/IKKε. A band of the appropriate molecular weight for IKK-i/IKKε (80 kDa) was seen in 4 of the 6 samples (Fig. 1A, left panel). Analysis of β-actin levels confirmed that loading was essentially equivalent. To verify the specificity, a duplicate blot was subjected to immunoblotting in the presence of 2 μg of cognate peptide (Fig. 1A, right panel). Detection of the 80 kDa band was completely eliminated with the addition of the peptide. Several lower molecular weight bands were detected, and these were also eliminated upon addition of the cognate peptide; therefore, they likely represent IKK-i/IKKε degradation products (data not shown). These findings indicate that IKK-i/IKKε is indeed expressed in multiple primary breast cancer specimens.

IKK-i/IKKε is expressed in DMBA-induced mammary tumors and in human breast cancer cell lines. We next tested extracts isolated from mammary tumors induced in female FVB mice by treatment with DMBA, which were primarily squamous cell carcinomas (SSC), with other tumors including a spindle cell carcinoma (SPC) and a microacinar tumor (MAC).[I] WCEs isolated from 7 mammary tumors as well as from the normal mammary glands from 3 age-matched parous FVB female control mice (FVB 4, FVB7, FVB8) were subjected to immunoblotting for expression of IKK-i/IKKε. Six of the seven tumors showed substantially elevated levels of IKK-i/IKKε compared to the mammary glands of non-treated control mice

[I] Nicolas Currier, Sandra E Solomon, Elizabeth G Demicco, Donny L F Chang, Marganit Farago, Haoqiang Ying, Isabel Dominguez, Gail E Sonenshein, Robert D Cardiff, Zhi J Xiao, David H Sherr, David C Seldin, Oncogenic signaling pathways activated in DMBA-induced mouse mammary tumors.

(Fig. 1B). Thus, IKK-i/IKKε expression is increased in many carcinogen-induced mouse mammary tumors.

To further characterize IKK-i/IKKε in breast cancer, we turned to human cell lines in culture. WCEs were prepared from the untransformed breast epithelial MCF-10F cell line and its DMBA-induced derivative line D3-1 (31) along with the breast cancer cell lines MDA-MB 231 and Hs578T, and subjected to immunoblot analysis for IKK-i/IKKε (Fig. 1C). A higher level of IKK-i/IKKε expression was observed in MDA-MB-231, Hs578T, and D3-1 cells compared to MCF-10F cells. MDA-MB 468 and T47D cells also displayed elevated IKK-i/IKKε levels (data not shown). To measure IKK-i/IKKε kinase activity, IKK-i/IKKε was immunoprecipitated from extracts prepared from MCF-10F, D3-1 and Hs578T cells and subjected to a kinase assay using as substrate either GST-WT-IκB-α or a mutant GST-2N-IκB-α protein (with phosphorylation sites Ser32 and Ser36 mutated to alanines) (Fig. 1D). Of the two transformed cell lines, Hs578T exhibited a higher level of IKK-i/IKKε activity than the D3-1 transformed cells; although, they appeared to contain almost equal levels of protein as judged by immunoblotting (Fig. 1D). Little or no IKK-i/IKKε activity was detectable in the MCF-10F cells, which also contained a much lower level of IKK-i/IKKε kinase protein (Fig. 1C and 1D). Thus, breast cancer cell lines contain an elevated level of IKK-i/IKKε.

IKK-i/IKKε expression is elevated in CK2α-expressing transgenic murine mammary tumors and glands. We next sought to determine whether mammary tumors in transgenic mice display elevated levels of IKK-i/IKKε, and selected the following mouse models: MMTV-CK2α, expressing the catalytic subunit of CK2, and the MMTV-c-*rel*, expressing the c-Rel NF-κB subunit. Approximately 30% of female MMTV-CK2α transgenic

mice developed a variety of mammary tumors at a median age of 23 months (22) and 31.6% of female MMTV-c-*rel* mouse developed one or more tumors at an average age of 19.9 months (30). Cytoplasmic extracts from mammary glands of age and pregnancy matched wild type (WT) mice were compared to those from tumors and histologically normal mammary glands of transgenic MMTV-CK2α (Fig. 2A) and MMTV-c-*rel* mice (Fig. 2B). Expression of IKK-i/IKKε protein was extremely low in the extracts from mammary glands of WT control animals. The MMTV-CK2α mouse tumor (7367T) displayed substantially higher levels than the WT-1 and WT-2 mouse mammary gland (Fig. 2A), while 4 of 5 tumors derived from MMTV-c-*rel* displayed a higher level of IKK-i/IKKε protein compared to the mammary glands of these two WT mice (Fig. 2B). As control for protein loading, the gel was stained with Coomassie, which indicated essentially equal loading. Interestingly, we noted that the histologically normal mammary glands of the MMTV-CK2α mice all appeared to contain substantial levels of IKK-i/IKKε as compared to the mammary glands of WT animals, whereas low levels were present in the histologically normal glands of the MMTV-c-*rel* transgenic mouse.

We have recently prepared a bitransgenic MMTV-CK2 x MMTV-c-*rel* mice, which exhibited approximately 50% incidence of breast tumor formation with the average age at onset of 20.8 months (S. Guo, unpublished observations). Histologically normal mammary glands of bitransgenic MMTV-CK2α x MMTV-c-*rel* mice were removed from the three animals that developed tumors and the levels of IKK-i/IKKε protein compared (Fig. 2C). Substantial IKK-i/IKKε expression was detected in cytoplasmic extracts of histologically normal mammary glands from these mice. The tumors from the same animal displayed somewhat higher IKK-i/IKKε levels. These findings are similar to the data obtained with the MMTV-CK2α mice, and suggest a role for CK2 in the induction of IKK-i/IKKε expression.

Ectopic CK2 elevates IKK-i/IKKε levels in NIH 3T3 fibroblasts, MCF-10F breast epithelial and HEK293T cells. To investigate the ability of CK2 to increase IKK-i/IKKε levels, isolated clones of CK2α stable lines (termed Clone 5 and Clone 6) in NIH 3T3 cells (10) were analyzed for expression IKK-i/IKKε compared to control NIH 3T3 cells (Fig. 3A, left panel). Higher levels of IKK-i/IKKε were seen in Clone 5 and Clone 6 as compared with the clones infected with the pBABE control viral vector. To examine the effects of ectopic CK2 expression in breast epithelial cells, MCF-10F cells, which have low levels of CK2 activity (9) and IKK-i/IKKε expression (Fig. 1B), were transiently transfected with vectors expressing HA-CK2α' and myc-CK2β, or parental empty expression vectors, as control. IKK-i/IKKε expression was increased compared to control MCF-10F cells transfected with empty vector DNA (Fig. 3A, right panel).

To further investigate the ability of CK2α to increase IKK-i/IKKε levels, cultures of HEK293T cells were transfected with vectors expressing either the catalytic CK2α' subunit as a HA-tagged protein (pRc/CMV-HA-CK2α') or the regulatory subunit CK2β as a c-Myc-epitope tagged protein (pRc/CMV-myc-CK2β), or a combination of both vectors. WCEs were subjected to immunoblotting for IKK-i/IKKε, and for the expression of the transfected CK2 subunits (Fig. 3B). Expression of either CK2 subunit alone or in combination led to a substantial increase in IKK-i/IKKε levels.

To assess whether CK2 acted at a pre-translational level to increase IKK-i/IKKε expression, semi-quantitative RT-PCR was performed using total RNA isolated from the HEK293T cells transfected as above (Fig. 3C). As a positive control, cells were transfected with a vector expressing IKK-i/IKKε. Ectopic expression of either CK2 subunit led to a substantial

induction of *ikk-i/ikke* mRNA levels, while analysis of *hmgb1*, which has been associated with estrogen responsive breast cancer (38-41), and *β-actin* showed no differential expression (Fig. 3C). These data strongly suggest a role for CK2 in the induction of IKK-i/IKKε.

Inhibition of CK2 activity decreases IKK-i/IKKε levels in breast cancer cells. As an initial test of the effects of CK2 on IKK-i/IKKε expression, breast cancer cells, which display high CK2 activity (9), were treated with apigenin, a selective inhibitor of CK2. Hs578T cells were incubated for 6 hours in the presence of either 25 or 50 μM apigenin or with an equal volume of carrier DMSO. RNA was isolated and subjected to semi-quantitative RT-PCR (Fig. 4A). As compared to control cells treated with DMSO, *ikk-i/ikke* mRNA expression decreased by 2.5-fold at 25 μM apigenin and by 2.9-fold with 50 μM apigenin (Fig. 4A) compared to the *β-actin* controls. Furthermore, treatment of Hs578T or D3-1or MDA-MB 231 breast cancer cells with 50 μM or 25 μM apigenin for 6 hours resulted in substantial decreases in IKK-i/IKKε protein levels (Fig. 4B). Since pharmacologic inhibitors may also affect other kinases, a vector expressing a kinase-inactive CK2α subunit, which functions as a competitive inhibitor with endogenous CK2α, was used (10, 33). Hs578T and D3-1 breast cancer cells were transfected with a vector expressing HA-tagged kinase inactive CK2α K68A or an empty vector control DNA, and cytoplasmic extracts analyzed. Hs578T and D3-1 cells expressing the kinase inactive CK2α K68A displayed lower levels of IKK-i/IKKε compared to control cells (Fig. 4C). Similar results were obtained using kinase inactive CK2α' K69A (data not shown). These findings indicate that CK2 activity induces expression of IKK-i/IKKε in breast cancer cells.

IKK-i/IKKε controls IκB-α turnover and NF-κB activity in breast cancer cells. Previous studies demonstrated IKK-i/IKKε phosphorylates IκB-α on Ser36 (24, 25). To

determine the *in vivo* effects of IKK-i/IKKε on steady state levels of IκB proteins in HEK293T cells, pCDNA3-FLAG-IKKε expression vector (IKKε) or empty vector pCDNA3 DNA (EV) were transfected into HEK293T cells. Cytoplasmic extracts, prepared 48 hours post transfection, were assessed for IκB proteins by immunoblotting. Ectopic IKK-i/IKKε expression caused a dramatic reduction in the total amount of cytoplasmic IκB-α (Fig. 5A). Only a modest drop in IκB-β levels was detected and no effect was seen on levels of IκB-ε. Furthermore, ectopic IKK-i/IKKε expression in HEK293T cells induced functional NF-κB activity as judged by NF-κB element driven reporter constructs (data not shown).

To verify a functional role of IKK-i/IKKε in regulation of NF-κB in breast cancer, a kinase inactive IKK-i/IKKε (IKKε K38A) was used, which serves as a dominant negative for IKK-i/IKKε activity. Her-2/neu receptor-driven mouse mammary tumor NF639 cells were used since CK2 activity in these cells had previously been implicated in the induction of NF-κB (10). NF639 cells were co-transfected with IKKε K38A expression vector or empty parental vector pCDNA3 in the presence of either E8-CAT or mut-E8-CAT, driven by wild type or mutant NF-κB sites, respectively. IKKε K38A caused a nearly 2-fold reduction in NF-κB activity (Fig. 5B, left panel). Confirmation of transfection was performed by immunoblotting for IKK-i/IKKε (data not shown). We next assessed the effects in Hs578T breast cancer cells of an increasing dose of the IKKε K38A expression on E8-CAT and mut-E8-CAT promoter activity (Fig. 5C). Expression of the kinase inactive IKK-i/IKKε resulted in a dose-dependent reduction of NF-κB activity. At the highest dose of IKKε K38A a nearly 5-fold reduction of E8-CAT activity was seen at 2 μg of IKKε K38A ($p<0.005$), reducing the activity of the WT E8-CAT almost to basal reporter activity seen with the mut-E8-CAT. Using a monoclonal FLAG-specific antibody in

immunoblot analysis of the remaining extracts confirmed the expression of IKKε K38A. Furthermore, when these extracts were analyzed for levels of IκB-α and IκB-β, a dramatic increase in the levels of IκB-α was observed with increasing doses of IKKε K38A. In contrast, no change was detected in levels of IκB-β (Fig. 5D). These results demonstrate IKK-i/IKKε plays a role in the constitutive activation of NF-κB in breast cancer cells and suggest IκB-α is the preferential IκB target.

IKKε K38A reduces activity of NF-κB-driven *cyclin D1* and *relB* promoters in breast cancer cells. To test the effects of the kinase inactive IKK-i/IKKε on natural promoters that are driven by NF-κB elements, co-transfection analysis was performed using the *cyclin D1* and *relB* promoters, which are each driven by two NF-κB elements (35, 36). Co-transfection of the kinase inactive IKKε K38A vector in Hs578T breast cancer cells resulted in a dose-dependent decrease in activity of the wild type *cyclin D1* luciferase reporter construct (-66 wt-Luc), whereas the mutant version (–66 mut-Luc) was largely unaffected (Fig. 6A). Similarly co-transfection of IKKε K38A with *p1.7 relB*-Luc *relB* promoter reporter construct, containing two WT NF-κB sites, reduced luciferase activity to that seen with a mutant construct, *p1.7 mut-relB*-Luc, containing mutations in the two identified NF-κB elements (Fig 6B). To ensure the effects were not cell type specific, D3-1 cells and MDA-MB 231 breast cancer cells were similarly co-transfected with either IKKε K38A or pCDNA3 in the presence of the WT *p1.7 relB*-Luc *relB* promoter construct (Fig. 6C). The kinase inactive IKK-i/IKKε caused a decrease in *relB* promoter activity in these two lines.

Next, we assessed whether inhibition of IKK-i/IKKε affects endogenous NF-κB driven gene expression. Hs578T cells were transiently transfected with IKKε K38A or parental vector.

Nuclear and cytoplasmic extracts were subjected to immunoblot analysis. Inhibition of IKK-i/IKKε led to reduced RelB and cyclin D1 expression (Fig. 6D). Similar results were obtained in D3-1 cells (Fig. 6D). Thus, IKK-i/IKKε plays an important role in control of functional NF-κB activity and its downstream targets in breast cancer cells.

IKKε K38A reduces breast cancer cell growth in soft agar and colony formation in matrigel. Since the aberrant expression of NF-κB cancer cells has been implicated in promoting anchorage independent growth (10, 42), Hs578T cells stably expressing IKKε K38A or pCDNA3 were used to assess the functional role of IKK-i/IKK-ε in this measure of transformed phenotype. Western blotting and reporter assays confirmed the presence of IKKε K38A and the ability of IKKε K38A stably expressing cells to reduce NF-κB driven reporters (data not shown). Cells were plated, in triplicate, and assayed for their ability to grow in soft agar. As seen in Figure 6E, expression of IKKε K38A resulted in a dramatic reduction in colony number, as judged by counting using a dissecting microscope. These data indicate that the induction of IKK-i/IKKε in breast cancer cells promotes transformed phenotype.

Lastly, we created stably expressing IKKε K38A NF639 cell lines to perform qualitative Matrigel invasion assays. Cells expressing pCDNA3 grow invasively in Matrigel whereas cells stably expressing IKKε K38A show a much less invasive phenotype (Fig. 6F). Inhibition of IKK-i/IKKε in these mammary cancer cells reduces invasiveness of Her-2/neu transformed tumor cells.

Discussion

Here, we demonstrate for the first time the functional role of IKK-i/IKKε in the aberrant activation of NF-κB in breast cancer and implicate CK2 in the induction of IKK-i/IKKε mRNA

and protein levels. IKK-i/IKKε expression was detected in primary human breast tumor specimens and cell lines, and in mammary tumors induced by DMBA treatment or that appeared in MMTV-CK2α and MMTV-c-*rel* transgenic animals and in MMTV-CK2α x MMTV-c-*rel* bitransgenic mice. We noted that histologically normal mammary glands of MMTV-CK2α and MMTV-CK2α x MMTV-c-*rel* bitransgenic mice also displayed high levels of IKK-i/IKKε expression, whereas mammary glands from MMTV-c-*rel* transgenic mice did not, suggesting a role for CK2 activity in regulation of IKK-i/IKKε. Of interest, elevated CK2α expression was seen in mammary tumors from DMBA-treated mice compared to the normal mammary glands of parous untreated control animals[I]. Ectopic expression of CK2 subunits induced IKK-i/IKKε levels. Conversely, inhibition of CK2 in breast cancer lines reduced endogenous IKK-i/IKKε levels. Previous studies from our lab have shown that a hallmark of breast cancer is the aberrant activation of NF-κB, which promotes tumor cell survival, growth and transformed phenotype (6, 10, 43). Consistent with these observations, inhibition of IKK-i/IKKε reduced the ability of breast cancer cells to grow in soft agar and form colonies in Matrigel. Thus, our findings implicate the induction of IKK-i/IKKε by CK2 as a new signaling pathway in the activation of NF-κB, and identify IKK-i/IKKε as a potential new chemotherapeutic target.

IKK-i/IKKε was first identified as a murine IKK-like protein with approximately 30% amino acid identity to IKKα and IKKβ in the kinase domain and expression inducible with LPS treatment (25). Subsequently, the human form of IKK-i/IKKε was also identified, cloned and shown to display a marked increase in activity, but not expression, upon treatment with the PKC activator PMA (24). In our studies, a lack of strict correlation was noted in the level of detectable protein compared to activity of IKK-i/IKKε in D3-1 and Hs578T cells, suggesting the possible

role of additional activation steps. Because of the sequence similarities between IKK-i/IKKε and IKKα and IKKβ, it was tested for its ability to phosphorylate IκB-α and found to specifically phosphorylate serine 36 of IκB-α and not serine 32. It was is believed that the IKK-i/IKKε kinase makes up part of an alternate IKK complex that lead to NF-κB/Rel activation. Recent studies have indicated that this complex does not play a role in toll-like receptor-mediated induction of NF-κB that has been implicated in LPS signaling since LPS treatment activated NF-κB/Rel in IKK-i$^{-/-}$ MEFs (44). However, these findings do not rule out a role for IKK-i/IKKε in NF-κB signaling in other systems. Our data shows that IKK-i/IKKε plays a role in aberrant constitutive NF-κB activation in breast cancer, suggesting that its activation may be organ or signal specific.

Our results confirm the ability of IKK-i/IKKε to phosphorylate IκB-α *in vitro*, and we also demonstrated its ability to regulate IκB-α levels and NF-κB activity in cells in culture. In contrast, the steady state levels of IκB-β (and those of IκB-ε) were much less affected by ectopic expression of IKKε or IKKε K38A *in vivo*; although, we have observed that IKK-i/IKKε can also phosphorylate IκB-β *in vitro* (data not shown). Overall, our findings suggest IκB-α is a preferential *in vivo* IκB target of IKK-i/IKKε. Consistent with previous work (24), we observed that the inhibition by IKKε K38A led to a decrease in activities of the NF-κB element driven promoters *cyclin D1* and *relB*. IKK-i/IKKε (in addition to other kinases) has recently been reported as a kinase capable of phosphorylating the p65 NF-κB subunit at Ser536, which may enhance its ability to bind to the IL-8 promoter (28). Thus, IKK-i/IKKε may act on multiple levels to regulate NF-κB activity. Recent studies also showed that IKK-i/IKKε had the ability to phosphorylate c-jun and affect downstream target genes, *mmp-3* and *mmp-13* (45), suggesting

IKK-i/IKKε involvement in multiple signaling pathways (25, 44-48). Of note, we have recently shown that c-jun regulates *relB* expression (36), thus it is conceivable that IKK-i/IKKε may work on multiple targets (NF-κB and AP-1) in regulating *relB* gene expression in breast cancer.

Studies from our lab have shown that TGF-β1 treatment reduces NF-κB activity in breast cancer cells (43). TGF-β1 treatment of NMuMG normal murine mammary epithelial cells leads to a detectable suppression of basal IKK-i/IKKε mRNA expression by 1 hour, and to a nearly complete suppression between 6-24 hours (49). Consistent with a role for CK2 in regulating IKK-i/IKKε, hepatocytes treated with TGF-β1 show reduced CK2 activity and stabilization of IκB-α levels (50). Breast cancer cell lines and tumor specimens have been shown to express elevated levels of CK2 (9, 10, 22, 23, 50). Taken together, these results suggest that IKK-i/IKKε may be affected by TGF-β1 treatment through its effects on CK2 activity, and suppression of IKK-i/IKKε expression may further enhance IκB-α stability. Overall, our studies identify a novel role for IKK-i/IKKε in NF-κB/Rel activation in breast cancer.

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References

1. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. Annu Rev Cell Biol 1994;10: 405-455.
2. Rayet B, Gelinas C. Aberrant rel/nfkb genes and activity in human cancer. Oncogene 1999;18: 6938-6947.
3. Smith MR, Greene WC. Identification of HTLV-I tax trans-activator mutants exhibiting novel transcriptional phenotypes. Genes Dev 1990;4: 1875-1885.
4. Duyao MP, Kessler DJ, Spicer DB, *et al.* Transactivation of the c-myc promoter by human T cell leukemia virus type 1 tax is mediated by NF kappa B. J Biol Chem 1992;267: 16288-16291.
5. Duyao MP, Buckler AJ, Sonenshein GE. Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter. Proc Natl Acad Sci U S A 1990;87: 4727-4731.
6. Sovak MA, Bellas RE, Kim DW, *et al.* Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. J Clin Invest 1997;100: 2952-2960.
7. Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ, Jr., Sledge GW, Jr. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. Mol Cell Biol 1997;17: 3629-3639.
8. Kim DW, Sovak MA, Zanieski G, *et al.* Activation of NF-kappaB/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis 2000;21: 871-879.
9. Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Traish AM, Mercurio F, Sonenshein GE. Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-kappaB in breast cancer. Cancer Res 2001;61: 3810-3818.

10. Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Sonenshein GE. Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells. Cancer Res 2002;62: 6770-6778.

11. Yamaoka S, Courtois G, Bessia C, *et al.* Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell 1998;93: 1231-1240.

12. Rothwarf DM, Zandi E, Natoli G, Karin M. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. Nature 1998;395: 297-300.

13. Barroga CF, Stevenson JK, Schwarz EM, Verma IM. Constitutive phosphorylation of I kappa B alpha by casein kinase II. Proc Natl Acad Sci U S A 1995;92: 7637-7641.

14. Lin R, Beauparlant P, Makris C, Meloche S, Hiscott J. Phosphorylation of IkappaBalpha in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. Mol Cell Biol 1996;16: 1401-1409.

15. Guerra B, Issinger OG. Protein kinase CK2 and its role in cellular proliferation, development and pathology. Electrophoresis 1999;20: 391-408.

16. Allende JE, Allende CC. Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. Faseb J 1995;9: 313-323.

17. Keller DM, Zeng X, Wang Y, *et al.* A DNA damage-induced p53 serine 392 kinase complex contains CK2, hSpt16, and SSRP1. Mol Cell 2001;7: 283-292.

18. Kato T, Jr., Delhase M, Hoffmann A, Karin M. CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response. Mol Cell 2003;12: 829-839.

19. Schwarz EM, Van Antwerp D, Verma IM. Constitutive phosphorylation of IkappaBalpha by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IkappaBalpha. Mol Cell Biol 1996;16: 3554-3559.

20. Pando MP, Verma IM. Signal-dependent and -independent degradation of free and NF-kappa B-bound IkappaBalpha. J Biol Chem 2000;275: 21278-21286.

21. McElhinny JA, Trushin SA, Bren GD, Chester N, Paya CV. Casein kinase II phosphorylates I kappa B alpha at S-283, S-289, S-293, and T-291 and is required for its degradation. Mol Cell Biol 1996;16: 899-906.

22. Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene 2001;20: 3247-3257.

23. Landesman-Bollag E, Song DH, Romieu-Mourez R, *et al.* Protein kinase CK2: signaling and tumorigenesis in the mammary gland. Mol Cell Biochem 2001;227: 153-165.

24. Peters RT, Liao SM, Maniatis T. IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex. Mol Cell 2000;5: 513-522.

25. Shimada T, Kawai T, Takeda K, *et al.* IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IkappaB kinases. Int Immunol 1999;11: 1357-1362.

26. Nomura F, Kawai T, Nakanishi K, Akira S. NF-kappaB activation through IKK-i-dependent I-TRAF/TANK phosphorylation. Genes Cells 2000;5: 191-202.

27. Pomerantz JL, Baltimore D. NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. Embo J 1999;18: 6694-6704.

28. Buss H, Dorrie A, Schmitz ML, Hoffmann E, Resch K, Kracht M. Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated

by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. J Biol Chem 2004;279: 55633-55643.

29. Bonnard M, Mirtsos C, Suzuki S, *et al.* Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. Embo J 2000;19: 4976-4985.

30. Romieu-Mourez R, Kim DW, Shin SM, *et al.* Mouse mammary tumor virus c-rel transgenic mice develop mammary tumors. Mol Cell Biol 2003;23: 5738-5754.

31. Calaf G, Russo J. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis 1993;14: 483-492.

32. Elson A, Leder P. Protein-tyrosine phosphatase epsilon. An isoform specifically expressed in mouse mammary tumors initiated by v-Ha-ras OR neu. J Biol Chem 1995;270: 26116-26122.

33. Penner CG, Wang Z, Litchfield DW. Expression and localization of epitope-tagged protein kinase CK2. J Cell Biochem 1997;64: 525-537.

34. Rawadi G, Zugaza JL, Lemercier B, *et al.* Involvement of small GTPases in Mycoplasma fermentans membrane lipoproteins-mediated activation of macrophages. J Biol Chem 1999;274: 30794-30798.

35. Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS, Jr. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol 1999;19: 5785-5799.

36. Wang X, Sonenshein GE. Induction of the RelB NF-kappaB subunit by the cytomegalovirus IE1 protein is mediated via Jun kinase and c-Jun/Fra-2 AP-1 complexes. J Virol 2005;79: 95-105.

37. Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 1987;7: 2745-2752.

38. Brezniceanu ML, Volp K, Bosser S, *et al.* HMGB1 inhibits cell death in yeast and mammalian cells and is abundantly expressed in human breast carcinoma. Faseb J 2003;17: 1295-1297.

39. Flohr AM, Rogalla P, Meiboom M, *et al.* Variation of HMGB1 expression in breast cancer. Anticancer Res 2001;21: 3881-3885.

40. He Q, Liang CH, Lippard SJ. Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. Proc Natl Acad Sci U S A 2000;97: 5768-5772.

41. Zhang CC, Krieg S, Shapiro DJ. HMG-1 stimulates estrogen response element binding by estrogen receptor from stably transfected HeLa cells. Mol Endocrinol 1999;13: 632-643.

42. Pianetti S, Guo S, Kavanagh KT, Sonenshein GE. Green tea polyphenol epigallocatechin-3 gallate inhibits Her-2/neu signaling, proliferation, and transformed phenotype of breast cancer cells. Cancer Res 2002;62: 652-655.

43. Sovak MA, Arsura M, Zanieski G, Kavanagh KT, Sonenshein GE. The inhibitory effects of transforming growth factor beta1 on breast cancer cell proliferation are mediated through regulation of aberrant nuclear factor-kappaB/Rel expression. Cell Growth Differ 1999;10: 537-544.

44. Kravchenko VV, Mathison JC, Schwamborn K, Mercurio F, Ulevitch RJ. IKKi/IKKepsilon plays a key role in integrating signals induced by pro-inflammatory stimuli. J Biol Chem 2003;278: 26612-26619.

45. Sweeney SE, Hammaker D, Boyle DL, Firestein GS. Regulation of c-Jun phosphorylation by the I kappa B kinase-epsilon complex in fibroblast-like synoviocytes. J Immunol 2005;174: 6424-6430.

46. McWhirter SM, Fitzgerald KA, Rosains J, Rowe DC, Golenbock DT, Maniatis T. IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. Proc Natl Acad Sci U S A 2004;101: 233-238.

47. Peters RT, Maniatis T. A new family of IKK-related kinases may function as I kappa B kinase kinases. Biochim Biophys Acta 2001;1471: M57-62.

48. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. Science 2003;300: 1148-1151.

49. Xie L, Law BK, Aakre ME, *et al.* Transforming growth factor beta-regulated gene expression in a mouse mammary gland epithelial cell line. Breast Cancer Res 2003;5: R187-198.

50. Cavin LG, Romieu-Mourez R, Panta GR, *et al.* Inhibition of CK2 activity by TGF-beta1 promotes IkappaB-alpha protein stabilization and apoptosis of immortalized hepatocytes. Hepatology 2003;38: 1540-1551.

Figure Legends

Figure 1. IKK-i/IKKε is expressed in breast cancer cells. *A,* Cytoplasmic extracts were prepared from the indicated human breast tumor specimens. Samples (50 μg) were used to prepare duplicate blots, which were subjected to immunoblotting with 2 μg of antibody specific for IKK-i/IKKε (Q-15, sc-9913) in the absence or presence of excess [2 μg] cognate IKK-i/IKKε peptide, as specific competitor. One blot was stripped and reprobed with an antibody against β-actin to confirm loading. *B,* Samples of WCEs (50 μg) from DMBA-induced mouse mammary tumors were subjected to immunoblotting for IKK-i/IKKε. Samples shown are all from a single gel. Coomassie blue stain (Coom. Blue) was used to control for equal loading. *C,* Left panel, Samples of WCEs (50 μg) prepared from human mammary epithelial cells MCF-10F, the DMBA-transformed D3-1 cell line and MDA-MB 231 (MB 231) breast cancer cells were subjected to immunoblotting for IKK-i/IKKε expression, and β-actin to control for loading. Right Panel, Whole cell extracts (25 μg) from MCF-10F, and Hs578T and MDA-MB 231 (MB 231) breast cancer cell lines were subjected to immunoblotting for IKK-i/IKKε and β-actin to control for loading. *D,* WCEs were prepared in kinase assay lysis buffer and precleared with Protein A/G Agarose beads and anti-goat secondary IgG HRP conjugated antibody. IKK-i/IKKε complexes were immunoprecipitated from 300 μg of protein using an N-terminal derived antibody. One third was used for a kinase assay on a GST-WT-IκB-α as substrate (KA), one third for a kinase assay on a GST-2N-IκB-α as substrate (KA) and one third for western blotting for IKK-i/IKKε (K-14, sc-5694, a C-terminal derived antibody) (IB). Immunoblotting of WCEs

shows endogenous levels of IKK-i/IKKε from MCF10F, D3-1 and Hs578T cells and β-actin to control for loading (WCE IB).

Figure 2. IKK-i/IKKε expression in mouse tumor models. Cytoplasmic extracts were prepared from mammary glands of age-matched wild type (WT) mice and histologically normal mammary glands from transgenic mice (N), and transgenic mouse tumor (T), as indicated. Samples (50 μg) were subjected to immunoblotting for IKK-i/IKKε expression. Coomassie blue (Coom. Blue) staining of duplicate gels was performed to confirm essentially equal loading. *A,* MMTV-CK2α transgenic mice; *B,* MMTV-c-*rel* transgenic mice; *C,* MMTV-CK2α X MMTV-c-*rel* bi-transgenic mice.

Figure 3. Ectopic CK2 expression induces IKK-i/IKKε in NIH 3T3 fibroblasts, MCF-10F breast epithelial and HEK293T cells. *A, Left panel,* Clones of NIH 3T3 cells infected with either pBABE-CK2α retrovirus expressing CK2α or a pBABE-GFP vector (pBABE) were isolated as described previously (10). WCEs (50 μg) were subjected to immunoblotting for either IKK-i/IKKε or β-actin, which confirmed equal loading. *Right panel,* MCF-10F breast epithelial cells were grown in P100 plates and transiently transfected with 10 μg pRc/CMV (EV) or 5 μg pRc/CMV-HA-CK2α' and 5 μg pRc/CMV-myc-CK2β (CK2α'/CK2β). WCEs were prepared and samples (100 μg) immunoblotted for IKK-i/IKKε. Duplicate blots were probed for HA and c-Myc to confirm expression of HA-CK2α' and Myc-CK2β. *B,* Cultures of HEK293T cells (in 6 well P60 dishes) were transfected with 1 μg of pRC/CMV-HA-CK2α' or pRC/CMV-myc-CK2β DNA alone or in combination and enough pCDNA3 (CMV driven) vector to make a total of 2 μg DNA. Protein extracts (50 μg) were subjected to immunoblot analysis for IKK-i/IKKε expression. To confirm expression of CK2α' and CK2β the blot was stripped and re-probed with

antibodies specific for HA and c-Myc, respectively. Equal loading was confirmed by probing the blot with a β-actin antibody. *C,* HEK293T cells were transfected as above with pRC/CMV-HA-CK2α' or pRC/CMV-myc-CK2β DNA alone or in combination, or with 2 μg of pCDNA3-FLAG-IKKε expressing FLAG-tagged IKK-i/IKKε, as a positive control. RNA was prepared using Trizol reagent. Samples (1 μg) were used for first strand cDNA synthesis, and a 2 μl aliquot (total 50 μl) used to perform RT-PCR analysis for *ikk-i/ikke*, *hmgb1* and *β-actin*. Two independent experiments were performed.

Figure 4. Inhibition of CK2 activity in breast cancer cells reduces IKK-i/IKKε expression. *A,* Hs578T breast cancer cells were grown in 6 well plates and treated with 25 μM or 50 μM apigenin for 6 hours. RNA was harvested using Trizol reagent. An RNA sample (1 μg) was used for first strand synthesis and a 2 μl aliquot was used for RT-PCR analysis of *ikk-i/ikke*, along with *hmgb1* and *β-actin* to control for loading. *B,* Hs578T, D3-1 and MDA-MB-231 breast cancer cells were treated with apigenin for 6 hours at a concentration of 25 μM or 50 μM (as indicated). WCEs were immunoblotted for IKK-i/IKKε expression. Blots were reprobed with a β-actin antibody to confirm equal loading. *C,* Hs578T and D3-1 cells, grown in 6-well plates, were transfected with 2 μg of either a kinase inactive HA-tagged CK2α construct (pRc/CMV-HA-CK2α K68A, labeled CK2α KI) or empty parental vector. Cells were harvested 16-24 hours post-transfection, lysed in RIPA buffer and samples of WCEs (60 μg, Hs578T or 75 μg, D3-1) subjected to immunoblotting for IKK-i/IKKε, HA (for CK2α KI), and β-actin as loading control.

Figure 5. IKK-i/IKKε controls IκB-α turnover and NF-κB activity. *A,* HEK293T cells, grown in 6-well plates, were transfected with 2 μg of empty vector pCDNA3 (EV) or pCDNA3-FLAG-IKKε (IKKε). Forty-eight hours post transfection, cytoplasmic extracts were prepared and

samples (20 μg) subjected to immunoblotting for IκB-α, IκB-β and IκB-ε expression. Blots were stripped and probed with β-actin to control for equal loading. A control blot was probed with a monoclonal FLAG antibody detecting FLAG-IKK-i/IKKε fusion protein. *B,* MMTV-Her2/*neu* NF639 cells were co-transfected, in triplicate, with 2 μg of pCDNA3-FLAG-IKKε K38A (IKKε K38A) or pCDNA3 (EV), 0.5 μg E8-CAT or mut-E8-CAT reporter construct and 0.5 μg of SV40-β-gal for normalization of transfection efficiency. The normalized relative activity of the E8-CAT to mut-E8-CAT is given. *C,* Hs578T cells were co-transfected, in triplicate, with 1 μg of either E8-CAT or mut-E8-CAT reporter construct along with 1 μg of SV40-β-gal, to normalize for transfection efficiency, and increasing concentrations of IKKε K38A [0.5, 1, 2 μg] while maintaining a 4 μg total DNA concentration with parental pCDNA3 vector. WCEs were prepared, normalized for transfection efficiency and subjected to CAT assays. Values for the mean +/- S.D. are presented. Significance was determined using the Student t-test. *D,* WCEs (20 μg) from the transfected Hs578T cells in part C were subjected to immunoblot analysis for the FLAG epitope, to confirm IKK-i/IKKε expression. The blots were stripped and re-probed with antibodies for IκB-α and IκB-β and subsequently for β-actin, which confirmed equal loading.

Figure 6. Inhibition of IKK-i/IKKε kinase in breast cancer cells reduces *cyclin D1* and *relB* expression, and transformed phenotype. *A,* Hs578T cells were co-transfected, in triplicate, with either 1.0 μg of *cyclin D1* –66 wt-Luc or –66 mut-Luc reporter construct along with 1.0 μg of SV40-β-gal and increasing concentrations of IKKε K38A [0.5, 1, 2.0 μg] while maintaining a 4 μg total DNA concentration with empty pCDNA3 vector. *B,* Hs578T cells were co-transfected, in duplicate, with 1.0 μg of either *p1.7 relB* or *p1.7 mut-relB* luciferase reporter construct along with 1.0 μg of SV40-β-gal and 2.0 μg of either IKKε K38A or pCDNA3. The average ± SEM is

presented. *C,* D3-1 (left panel) cells were co-transfected, in duplicate, with either 1.0 μg *p1.7 relB* luciferase or *p1.7 kB mut-relB* luciferase reporter construct along with 1.0 μg of SV40-β-gal and 2.0 μg of either pCDNA3-FLAG-IKKε K38A or pCDNA3. Luciferase activity was measured and normalized to β-gal activity. The average ± SEM is presented. Alternatively, MDA-MB 231 (right panel) cells were co-transfected, in triplicate, and processed as above. Standard deviation (S.D.) was calculated and significance determined using the Student t-test. *D,* Hs578T and D3-1 cells in P100 plates were transfected with 10 μg of pCDNA3-FLAG-IKKε K38A (IKKε K38A) or pCDNA3 (EV). Cytoplasmic, nuclear or WCEs were isolated (as indicated), and samples (20 μg) subjected to immunoblotting for RelB and cyclin D1 expression. *E,* Hs578T breast cancer cells stably expressing IKKε K38A or pCDNA3 were plated, in triplicate, in soft agar. Following incubation at 37^0C for 16 days, the plates were stained with crystal violet to visualize the cells and photographed using a Kodak digital camera. Colonies are shown at 3.2 X magnification and counted at 8X magnification in 3 randomly selected fields of view. *F,* NF639 cells were plated in matrigel in 12-well plates (5,000 cells per well), in triplicate, and assessed for their ability to grow between 3 and 6 days. The plates were photographed using an Orca ER camera at 5X magnification in a Zeiss Axiovert 200M microscope.

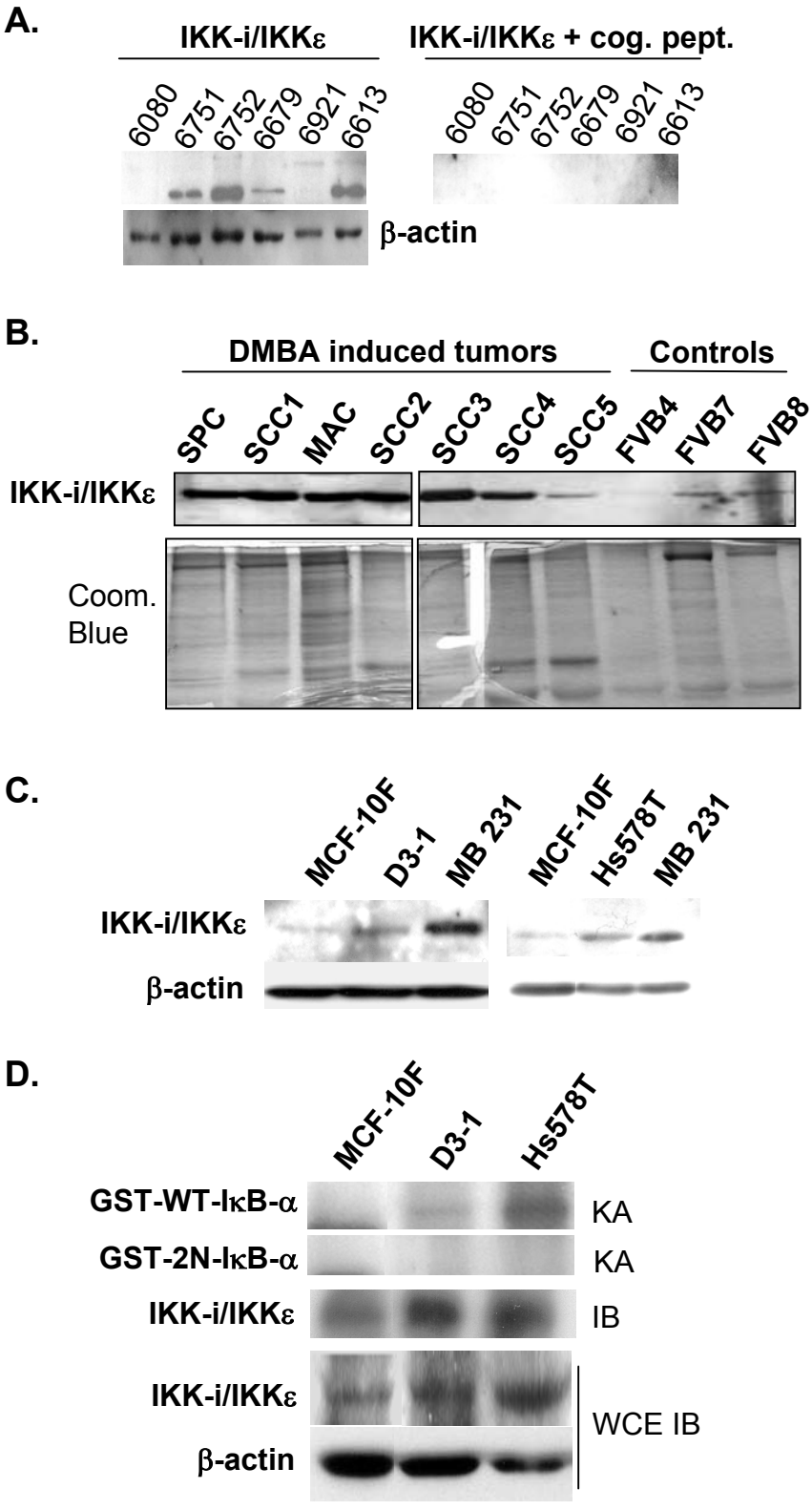
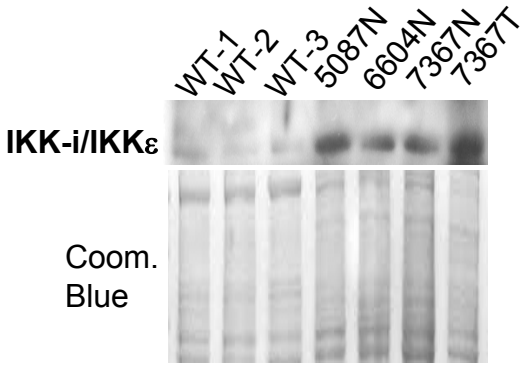
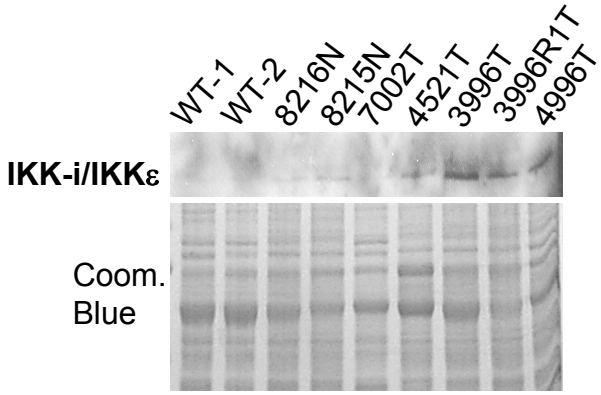


Figure 1, Eddy et al.

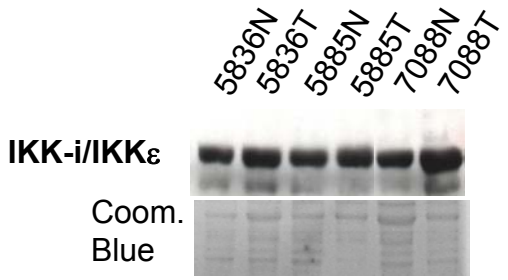
A. MMTV-CK2 transgenic mice



B. MMTV-*c-rel* transgenic mice



C. MMTV-CK2 X MMTV-c-*rel* mice



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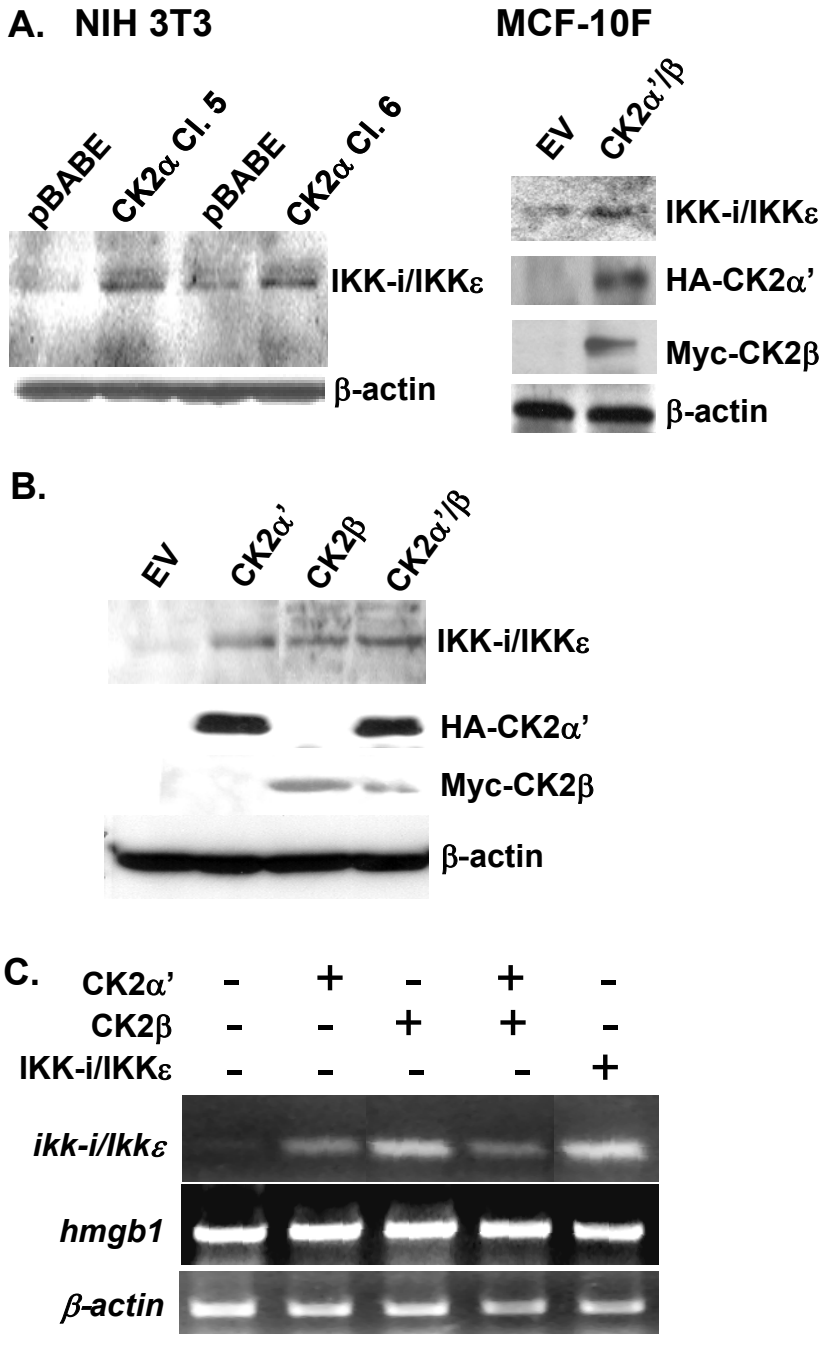

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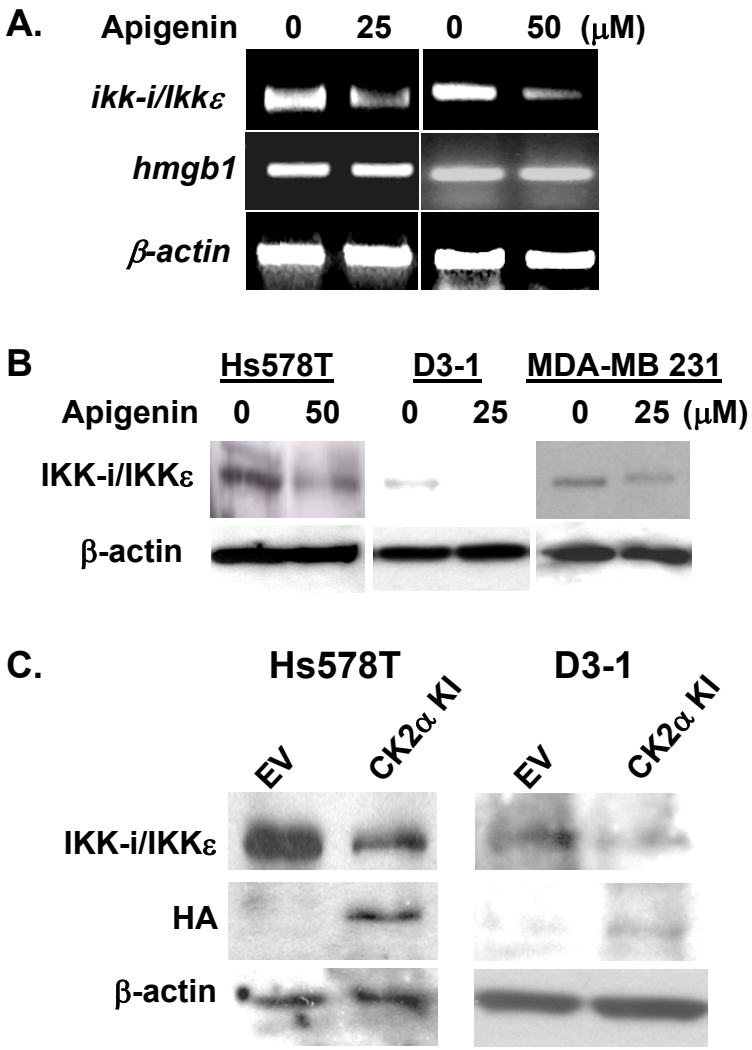
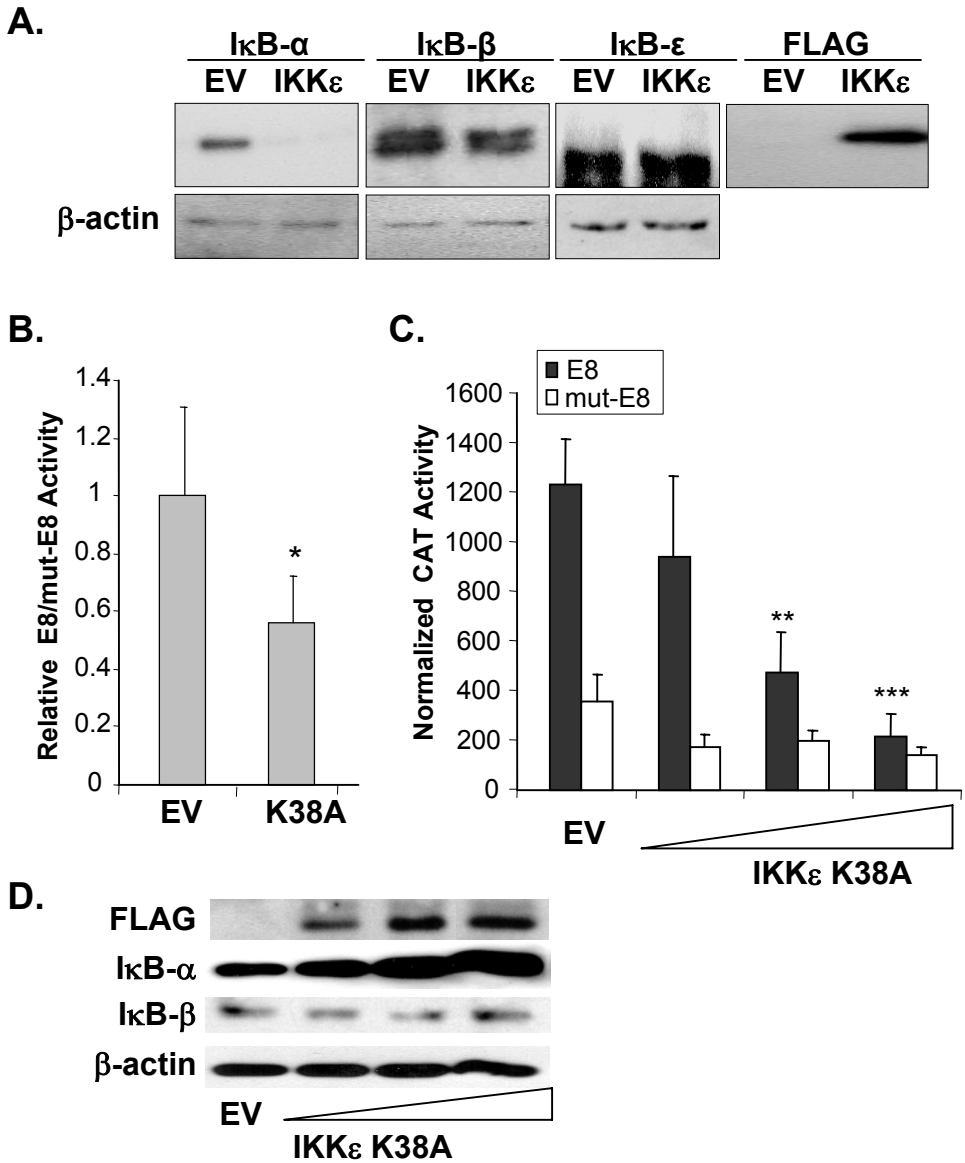


Figure 4, Eddy et al.



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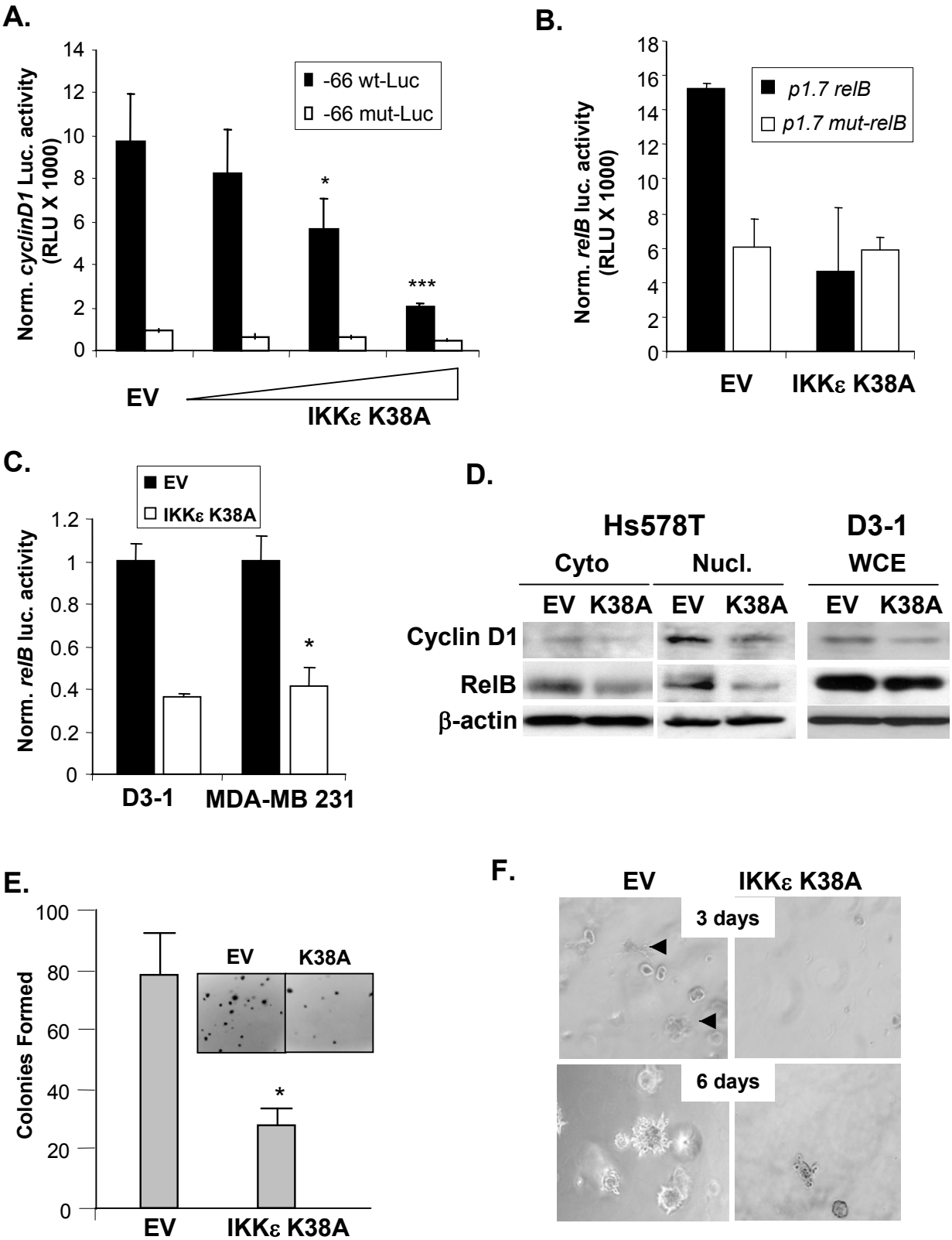

Figure 6, Eddy et al.

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Protein kinase CK2 in mammary gland tumorigenesis

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Protein kinase CK2 is a ubiquitous and evolutionarily conserved serine/threonine kinase that is upregulated in many human cancers and can serve as an oncogene in lymphocytes. Recently, we have demonstrated that CK2 potentiates Wnt/β-catenin signaling in mammary epithelial cells. To determine whether CK2 overexpression contributes to mammary tumorigenesis, we have performed comparative studies of human and rat breast cancer specimens and we have engineered transgenic mice with dysregulated expression of CK2α in the mammary gland. We find that CK2 is highly expressed in human breast tumor specimens and in carcinogen-induced rat mammary tumors. Overexpression of CK2α in the mammary gland of transgenic mice, under control of the MMTV-LTR, causes hyperplasia and dysplasia of the female mammary gland. Thirty per cent of the female MMTV-CK2α transgenic mice develop mammary adenocarcinomas at a median of 23 months of age, often associated with Wnt pathway activation, as evidenced by upregulation of β-catenin protein. NF-κB activation and upregulation of c-Myc also occur frequently. Thus, in mice, rats, and humans, dysregulated expression of CK2 is associated with and is capable of contributing to mammary tumorigenesis. Targeted inhibition of CK2 could be useful in the treatment of breast cancer. *Oncogene* (2001) **20,** 3247–3257.

Keywords: casein kinase II; CK2; transgenic mice; breast cancer; NF-κB; β-catenin

Introduction

Protein kinase CK2 (formerly known as casein kinase II), is a tetrameric serine-threonine kinase constituted of two α or α′ catalytic subunits of 42–44 and 38 kDa, respectively, and two β regulatory subunits of 28 kDa. It is remarkably conserved throughout evolution (Heller-Harrison *et al*., 1989; Seldin and Leder, 1995) and ubiquitously found in all eukaryotic cells, indicative of a vital cellular role for this protein. CK2 is required for cell cycle progression in yeast (Glover, 1998), and may have a role in G_2 checkpoint regulation (Toczyski *et al*., 1997). CK2 is also involved in cell cycle progression in mammalian cells (Orlandini *et al*., 1998; Pepperkok *et al*., 1994); we have shown that CK2 phosphorylates HSIX1, a homeobox protein with a G_2 cell cycle checkpoint function in human breast cancer cells (Ford *et al*., 2000). Targeted disruption of the CK2α′ subunit in mice reveals an indispensable role for this subunit in male germ cells where it is preferentially expressed (Xu *et al*., 1999). In general, CK2 is elevated in proliferative tissues: its expression is high in embryonic tissues (Issinger, 1993; Mestres *et al*., 1994), regenerating liver (Pancetti *et al*., 1996; Perez *et al*., 1988), and also in the proliferative cell layer at the base of the crypt in colonic mucosa (Munstermann *et al*., 1990). The mechanisms by which CK2 regulates cell proliferation remain unclear, as CK2 has hundreds of substrates in cells and affects many critical cellular growth pathways (reviewed in Allende and Allende, 1995; Guerra and Issinger, 1999; Pinna and Meggio, 1997). Like HSIX1, many other transcription factors can be regulated by CK2 phosphorylation, as has been demonstrated for c-Myc, c-Myb, AP-1, UBF, homeodomain proteins, steroid hormone receptors, and others. The p65 subunit of NF-κB is a direct CK2 substrate (Bird *et al*., 1997), and CK2 also regulates NF-κB through phosphorylation of its inhibitory subunit IκB (Janosch *et al*., 1996; Lin *et al*., 1996; Pando and Verma, 2000; Schwarz *et al*., 1996). In *Drosophila*, phosphorylation of the IκB homolog cactus by CK2 is required for wild type axis formation (Liu *et al*., 1997). Recently, CK2 was shown to interact with elements of the wingless/Wnt signaling pathway in insect cells (Willert *et al*., 1997) and we have demonstrated that CK2 is a positive regulator of Wnt signaling in mouse mammary epithelial cells (Song *et al*., 2000). Activation of this pathway by the mouse mammary tumor virus

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(MMTV) leads to breast cancer in mice (Nusse and Varmus, 1982). In many human cancers, activation of the Wnt signaling pathway occurs through inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene or mutation of the transcriptional cofactor β-catenin (Morin *et al*., 1997; Polakis, 1999; Roose and Clevers, 1999).

CK2 is also upregulated in cancer. This was reported in lymphoblastoid cells in bovine tropical theileriosis, a parasite-induced pseudoleukemia (ole-MoiYoi *et al*., 1993). In human malignancies, CK2 is elevated in lung, prostate, head and neck cancers (Ahmed, 1994; Daya-Makin *et al*., 1994; Faust *et al*., 1996; Gapany *et al*., 1995; Munstermann *et al*., 1990), malignant melanoma (Mitev *et al*., 1994), and in leukemias (Pena *et al*., 1983; Roig *et al*., 1999). CK2 overexpression may be a contributor to the molecular pathogenesis of these malignancies, as overexpression of CK2 in lymphocytes in transgenic mice leads to lymphoma (Seldin and Leder, 1995). CK2 transformation requires the participation of other oncogenic events, as lymphoma develops slowly in clonal cells in transgenic mice. Lymphomagenesis is accelerated by the presence of c-*myc* or *tal-1* transgenes or by loss of the *p53* tumor suppressor gene (Kelliher *et al*., 1996; Landesman-Bollag *et al*., 1998; Seldin and Leder, 1995). In human breast cancer, immunohistochemistry has demonstrated strong nuclear staining of the CK2α subunit in breast tumor compared to normal tissue (Munstermann *et al*., 1990). This finding, along with the functional role in the Wnt/β-catenin pathway and in regulation of HSIX1 in breast cancer cells, suggested that CK2 might play a causative role in mammary tumorigenesis. To investigate this hypothesis, we examined CK2 expression in human and rat breast tumors. We then engineered transgenic mice overexpressing the α subunit of CK2 in the mammary gland and studied the consequences of dysregulated CK2 expression in that tissue.

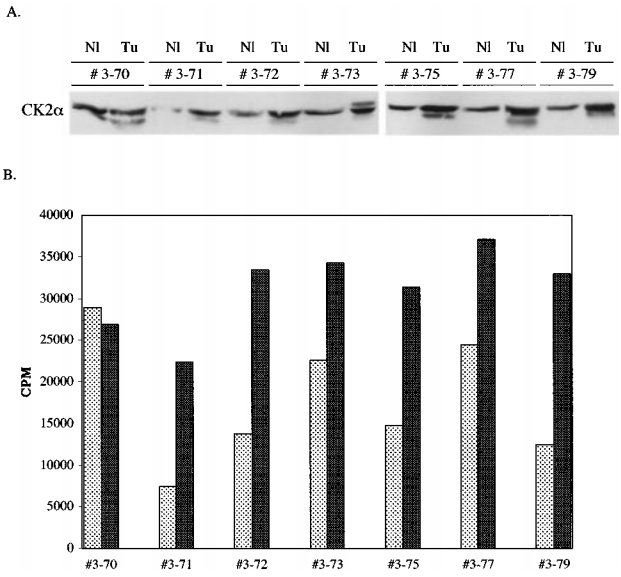

Figure 1 Protein kinase CK2 expression and activity in carcinogen-induced mammary tumors in rats. (**a**) One hundred micrograms of protein extracted from paired normal mammary glands (N1) and mammary tumors (Tu) from rats treated with DMBA were subjected to Western blotting for CK2; equal loading was confirmed by Ponceau S staining (not shown). CK2 expression is elevated in six out of seven tumor samples. (**b**) CK2 kinase activity: equal amounts of protein lysates were assayed in duplicate for phosphorylation of a specific CK2 substrate peptide (Kuenzel *et al*., 1987). Data are expressed as the difference of the mean counts of the kinase reaction carried out without the specific peptide subtracted from the mean with peptide; error bars cannot be displayed for the difference of means but were generally $<10\%$ for the means themselves. In six out of seven pairs, a marked increase in CK2 kinase activity in tumors (dark bars) is seen compared to normal mammary tissue (light bars). The overall difference in kinase activity between mammary glands and tumors is statistically significant ($P<0.003$)

Results

CK2 is highly expressed in rat and human breast tumors

As a first step in determining whether protein kinase CK2 plays a role in the pathogenesis of mammary tumorigenesis, we measured CK2 catalytic protein subunit expression and activity in rat carcinogen-induced breast tumors. Rats treated with a single oral dose of 7,12-dimethylbenz[*a*]anthracene (DMBA) develop mammary tumors 7–20 weeks later (Huggins *et al*., 1961; Rogers and Wetsel, 1981). Proteins extracted from snap frozen tumors or control pooled normal mammary tissue from the same rats were analysed as matched pairs of samples. In six out of seven pairs, CK2 protein is upregulated in the tumors; densitometry analysis showed an average increase of 2.7-fold (Figure 1a). The differing mobility of the bands corresponds to the 42 kDa CK2α and the 38 kDa CK2α' subunits, both of which are recognized by the polyclonal rabbit antibody. Recombinant CK2 holoenzyme was run in parallel, and the recombinant α and α' bands comigrated with the rat CK2 bands (not shown). Western blot analysis of the CK2β subunit revealed no significant change in band intensity between normal and malignant tissue (not shown).

We measured kinase activity in these samples using an assay based on phosphorylation of a specific CK2 substrate peptide, RRREEETEEE (Kuenzel *et al*., 1987). Samples were assayed in duplicate, and [γ-^{32}P]GTP was employed as a phosphate donor to further ensure specificity of the assay, since CK2 is one of the few enzymes capable of utilizing GTP. The same six out of seven pairs exhibited increased kinase activity in the tumors, with a mean increase of twofold (Figure 1b). Thus both CK2 protein levels and kinase activity are comparably increased in rat mammary tumors.

We then examined CK2 levels in human breast cancer. Ten anonymous human breast tumor specimens and a non-malignant control reduction mammoplasty specimen were obtained from a tumor bank at Boston University School of Medicine, with approval of the Institutional Review Board. All breast tumor specimens exhibited abundant expression of the catalytic

subunits compared to the non-malignant mammoplasty specimen (Figure 2a). CK2 kinase activity was determined in the seven samples in which there was adequate material for duplicate measurements. All of these seven samples had more CK2 activity than the control, and on average the increase was more than 10-fold (Figure 2b). Again, the varying mobility of these bands may reflect the relative amounts of CK2α and CK2α'; furthermore, in transformed cells, a faster migrating form of CK2α has been observed (Roig *et al*., 1999). Thus, in both carcinogen-induced breast tumors in a rodent model system and in human breast tumors, CK2 protein levels and activity are elevated, suggesting that CK2 may play a role in mammary tumorigenesis.

Transgenic expression of CK2α in the mouse mammary gland

To determine whether CK2 overexpression plays a causative role in mammary tumorigenesis, we engineered transgenic mice to overexpress the α catalytic subunit of CK2 in the mammary gland using an MMTV-LTR transgene vector (Stewart *et al*., 1984). FVB/N mice were utilized. Integration of the construct into the genome of potential founders was assessed by Southern blot using the CK2α cDNA as a probe. Four founders successfully passed the transgene through the germline and their progeny were used for this study. A transgene-specific RT–PCR assay, using a 5′ CK2α cDNA sense primer and a 3′ SV40 poly(A)$^+$ antisense primer, was employed to assess the expression of the transgene in mouse organs. Virgin, pregnant, and involuting mammary glands all express the transgene (Figure 3). Weak bands are also seen in thymus, small intestine, and salivary gland; this glandular pattern of expression has been seen with other MMTV transgenes (Sinn *et al*., 1987). Both spliced and unspliced transgenic CK2α PCR products (arrows, Figure 3) were gel-purified and sequenced to confirm their identity.

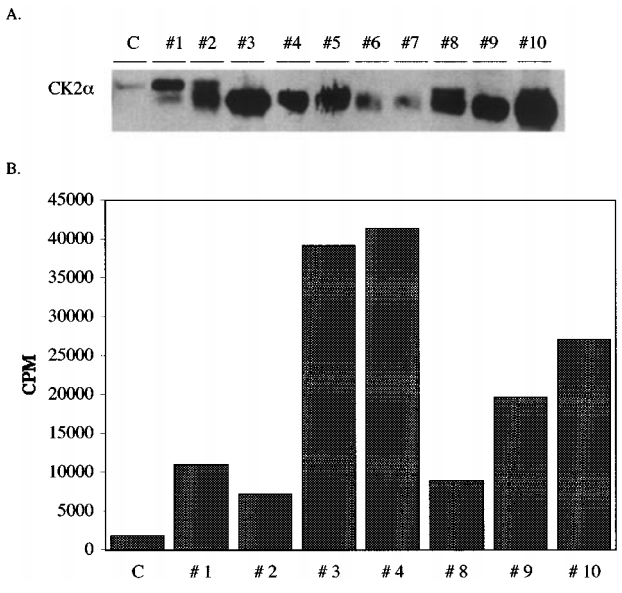

Figure 2 Protein kinase CK2 expression and activity in human breast tumors. (**a**) Representative Western blot of tissue extracts with 50 μg of protein lysate in each lane. Breast tissue obtained from a reduction mammoplasty was used as a control and compared with 10 breast cancer specimens; increased CK2 protein as seen in all 10 specimens. Equal loading was obtained as in Figure 1. (**b**) CK2 kinase activity: equal amounts of protein lysates were assayed for phosphorylation of the specific CK2 peptide as described in Figure 1b; insufficient material was available for analysis from samples 5–7. All breast tumor specimens assayed have increased levels of CK2 activity

MMTV-CK2 mice develop hyperplasia and dysplasia in the post-lactating mammary gland

MMTV-CK2α transgenic mice develop and breed normally. To promote transgene expression from the hormone-dependent MMTV-LTR, female mice were continuously bred and pups were removed after 7 days of lactation. Mammary glands from female mice at several stages of mammary gland development were taken for histological analysis. Histological abnormalities were observed in half of the MMTV-CK2α female transgenic mice (14 out of 27 mice examined). In three mice, pubertal mammary gland development was incomplete or retarded, with less than 20% of the mammary fat pad being filled with glandular tissue, although ultimately the females were able to produce adequate milk. Incomplete mammary gland regression was observed in eight mice whose mammary glands were harvested 2–12 months after the last pregnancy. Importantly, seven mice developed a variety of dysplastic lesions that are rarely seen in wild type FVB/N females, and are considered to be premalignant (Cardiff, 1996). These included areas of dysplastic alveoli and squamous metaplasia, commonly seen with areas of inflammation in the stroma and the epithelium

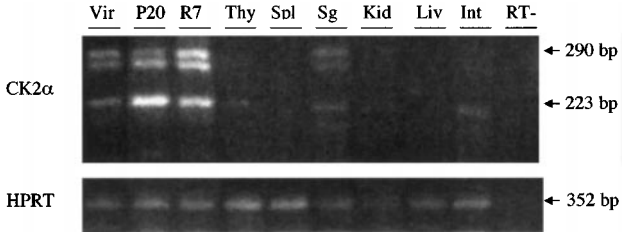

Figure 3 MMTV-LTR-driven CK2α transgene expression in transgene mice. Five to 10 micrograms of total RNA derived from organs obtained from virgin, full-term pregnant and 7 days post-lactation transgenic female mice were subjected to DNAse treatment, followed by RT–PCR. Transgene-specific oligonucleotides encompass a 67 bp splicing region in the SV40 polyA tail. Thus, unspliced mRNA yields a 290 bp band while the spliced mRNA yields a 223 bp band. Virgin, pregnant and post-lactational mammary glands express the transgene. CK2α transgenic message is also detected in thymus, salivary glands and small intestine. *hprt* amplification confirms the integrity of the reverse transcription reaction (Johnson *et al*., 1988). Vir: virgin, P20: 20th day of gestation, R7: 7 days post-lactation, thy: thymus, spl: spleen, sg: salivary gland, kid: kidney, liv: liver, int: small intestine, RT–: no reverse transcriptase added

(Figure 4). Ductal ectasia was frequently seen in regressed mammary glands, as has been reported with other oncogenic transgenes (Webster *et al*., 1998). Thus, mice with dysregulated expression of CK2α in the mammary gland frequently exhibit abnormalities of mammary gland development and regression, and inflammatory and preneoplastic lesions.

MMTV-CK2α mice develop late-onset mammary adenocarcinomas

A cohort of 56 transgenic female mice was observed for over 2 years. Thirty per cent of the mice developed mammary tumors at a median age of 23 months. In addition, over 35% of the mice developed non-mammary malignancies, including lung tumors and lymphomas. The incidence of non-mammary tumors was very similar to that reported for wild type mice of the same FVB/N strain (Mahler *et al*., 1996), and thus most likely does not result from an effect of the transgene (Table 1). No spontaneous mammary tumors were reported in the wild type cohort (Mahler *et al*., 1996), and while spontaneous mammary tumors do occur with a very low incidence (<1%) in FVB/N mice (RD Cardiff, unpublished observation), all four of our founder lines developed breast tumors. Thus, mammary tumor development in the transgenic cohort is likely due to transgene CK2α expression rather than an insertional event. The tumors generally arose as solitary masses in single mammary glands, although multiple tumors developed synchronously in separate glands in six mice. Tumors and other mammary glands and organs were harvested for histological and molecular analyses. All the tumors were adenocarcinomas, which could be further classified into a variety of subtypes. The most common histologic subtypes were glandular and papillary adenocarcinomas, in seven tumors each (Figure 5). Four were classified as adenosquamous carcinomas (keratoacanthomas) and two as scirrhous carcinomas. One tumor consisted of large cells similar to those seen with expression of a c-*myc* transgene in the mammary gland, and one had the solid cord-like pattern of tumors as seen arising through transgenic expression of *int-2* (Cardiff and Wellings, 1999). Some tumors exhibited mixed patterns with classical adenocarcinoma in some regions and the cellular connective tissue stroma characteristic of scirrhous carcinomas in others.

Six tumors from four mice had histologic features of spindle cell carcinomas. The spindle cell pattern suggested that these may have arisen from the mammary stroma or mesenchyme rather than from the epithelium. To identify the cell of origin, immunohistochemical staining was carried out using antibodies to vimentin as a marker for mesenchymal cells and antibodies to cytokeratins 8 and 14 and smooth muscle actin to identify cells of epithelial or myoepithelial origin. The spindle cell tumors stained negligibly for vimentin but were strongly positive for the cytokeratins and smooth muscle actin, identifying these tumors to be of epithelial origin (Figure 6). This

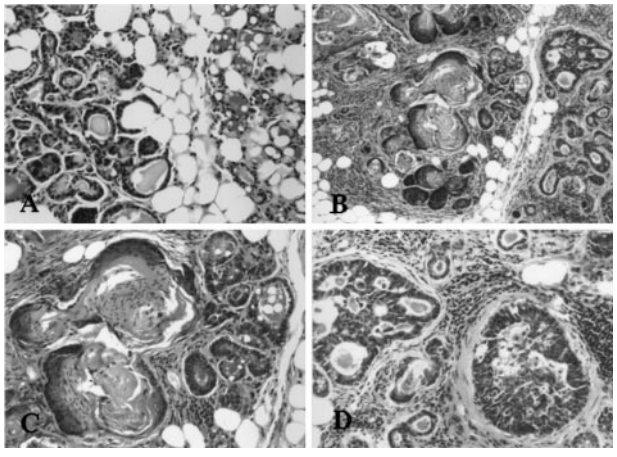

Figure 4 Mammary gland dysplasia in regressed, multiparous MMTV-CK2α transgenic mice. Hematoxylin and eosin-stained sections reveal that mammary glands from female transgenics frequently present with foci of dysplasia. (**a**) This field contains a dysplastic region on the left hand side and normal-appearing regressing mammary gland on the right hand side of the image. Compare the size and density of the nuclei in the dysplastic alveolar nodules on the left with the normal epithelium on the right. (**b**) This field includes both alveolar and squamous nodules, with the squamous nodules enlarged in (**c**) and the alveolar nodules enlarged in (**d**). Note that all fields of dysplasia are associated with extensive fibrosis and inflammation

Table 1 Tumor incidence in female MMTV-CK2α transgenic mice compared with wild type FVB/N at 24 months of age

	MMTV-CK2α transgenic mice[a] (%)	*Wild type*[b] (%)
Breast tumors	30	0
Lung tumors	21	28
Lymphomas	14	13
Other tumors or undiagnosed	17	21

[a]Fifty-six females from the MMTV-CK2α transgenic mouse colony (FVB/N background strain) were observed for tumor incidence. [b]Seventy-one wild type FVB/N female mice were monitored for tumor incidence as reported by the National Institute for Environmental Health Sciences (Mahler *et al*., 1996). For this comparison, only histological malignant lesions were scored as tumors; adenomas and other benign lesions were not included

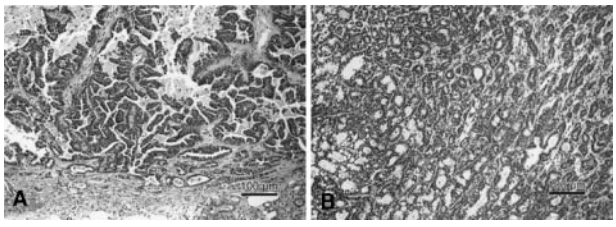

Figure 5 Representative histopathologies of breast tumors developing in aging MMTV-CK2α transgenic female mice after multiple cycles of pregnancy and regression. All the breast tumors are adenocarcinomas, presenting with a variety of patterns, including papillary adenocarcinomas, characterized by a frond-like pattern with central fibrovascular stalks covered by neoplastic epithelium (**a**), or pure glandular adenocarcinomas. (**b**). Hematoxylin and eosin staining (100×)

result was further confirmed by immunofluorescence staining of two cell lines derived from the spindle cell tumors, which stained positively with a broad specificity anti-keratin antibody, similar to control MCF-7 human mammary epithelial breast tumor cells (not shown). Thus, these spindle cell adenocarcinomas have arisen through the transformation of epithelial cells into cells with histological features of mesenchymal cells; this phenomenon has been described as an epithelial to mesenchymal transition (EMT). Tumors with features of EMT have been reported to have increased invasiveness (Birchmeier *et al*., 1996). Indeed, the spindle cell transgenic breast tumors behave more aggressively than other MMTV-CK2α mammary tumors, growing rapidly in culture or upon transplantation into subcutaneous tissues of syngeneic recipients.

Expression of CK2 and candidate downstream targets in transgenic mammary tumors

CK2 protein expression and kinase activity in the tumors was determined as previously done in the rat and human breast tumor specimens; these assays measure the sum of transgenic and non-transgenic catalytic CK2. Grossly normal mammary gland from transgenic mice that developed mammary tumors were paired with the mammary tumors as controls. Twelve pairs of tumors and controls were analysed, and all exhibit higher levels of CK2α protein in the tumors. Four pairs are depicted in a representative Western blot (Figure 7a); CK2 expression can be seen in the normal glands with longer exposures (not shown). We also find upregulation of CK2 in transformed cell lines derived from these tumors, indicating that this is a property of the tumor cells themselves. CK2 kinase activity was also elevated in the tumors, averaging sixfold in six pairs assayed (Figure 7b). Similarly to rat tissues, no significant change in CK2β expression was detected (not shown).

The mammary tumors in the transgenic mice occurred in older mice, suggesting a requirement for multiple additional molecular events for complete mammary epithelial cell transformation. Some molecular alterations may be direct consequences of CK2α misexpression, while others may occur independently. Our recent observations implicate CK2 as a positive regulator of the Wnt/β-catenin pathway in mammary epithelial cells (Song *et al*., 2000). To determine whether the CK2α transgenic tumors harbor an activated Wnt/β-catenin signaling pathway, β-catenin levels were assessed by Western blot analysis of tumor tissue extracts; a representative Western blot is shown (Figure 8a). Overall, six of 11 breast tumors tested exhibited elevated levels of β-catenin compared to non-malignant transgenic mammary tissues, indicative of an activated Wnt/β-catenin pathway.

CK2 phosphorylates IκB and promotes its degradation, regulating basal as weel as inducible levels of nuclear NF-κB (Janosch *et al*., 1996; McElhinny *et al*., 1996; Pando and Verma, 2000; Schwarz *et al*., 1996).

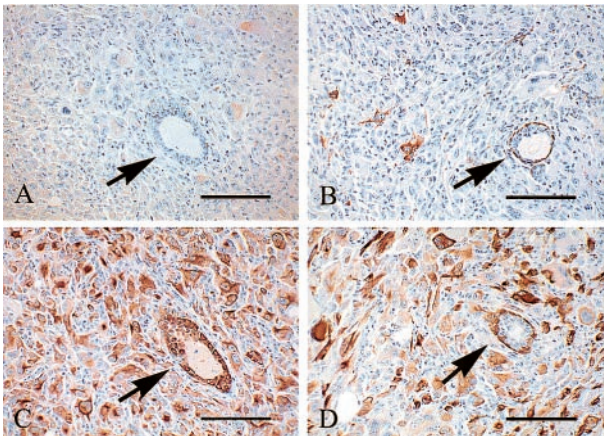

Figure 6 Immunohistochemistry of a transgenic spindle cell carcinoma. Comparable fields are shown to illustrate the mixture of tumor giant cells, fusiform spindle cells and scattered glands (arrows). Note that the tumor cells show relatively low levels of staining for vimentin (**a**). Anti-smooth muscle actin stains a well-defined myoepithelial layer in the glands (**b**, arrow) and scattered tumor cells. Anti-cytokeratin 8 stains the luminal epithelium in the glands (**c**, arrow) and many of the spindle cells and tumor giant cells. Anti-cytokeratin 14, a marker of myoepithelial cells, identifies both the myoepithelium and the spindle cell population but not the luminal cells of the glands (**d**). These results indicate that the spindle cell tumors are derived from cells of epithelial origin. Scale bars = 100 μm

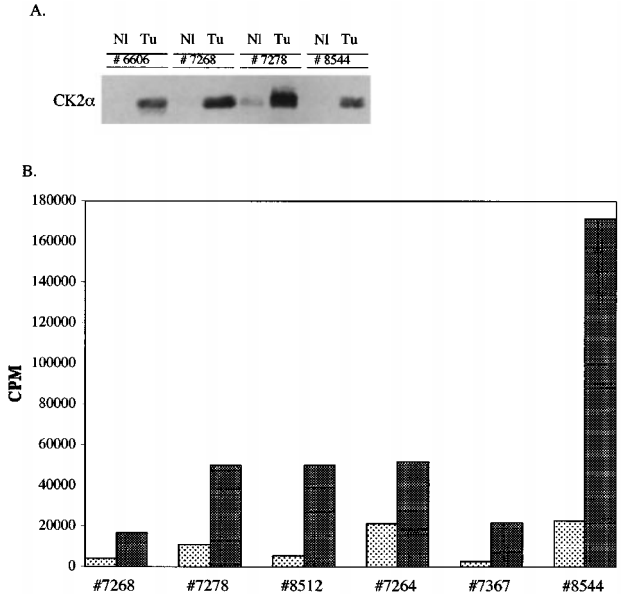

Figure 7 Protein kinase CK2 expression and activity in transgenic female MMTV-CK2α breast tumors. (**a**) One hundred micrograms of protein extracted from paired normal mammary glands (N1) and breast tumors (Tu) of transgenic mice were subjected to Western blotting for CK2. A representative blot demonstrates that the tumors have increased levels of CK2 protein. (**b**) CK2 kinase activity of the protein extracts was measured with the CK2 peptide kinase asssay as in Figure 1. Although kinase activity in the tumors was variable, the overall difference in kinase activity between mammary glands and tumors was statistically significant ($P < 0.023$). Normal mammary tissue: light bars; breast tumors: dark bars

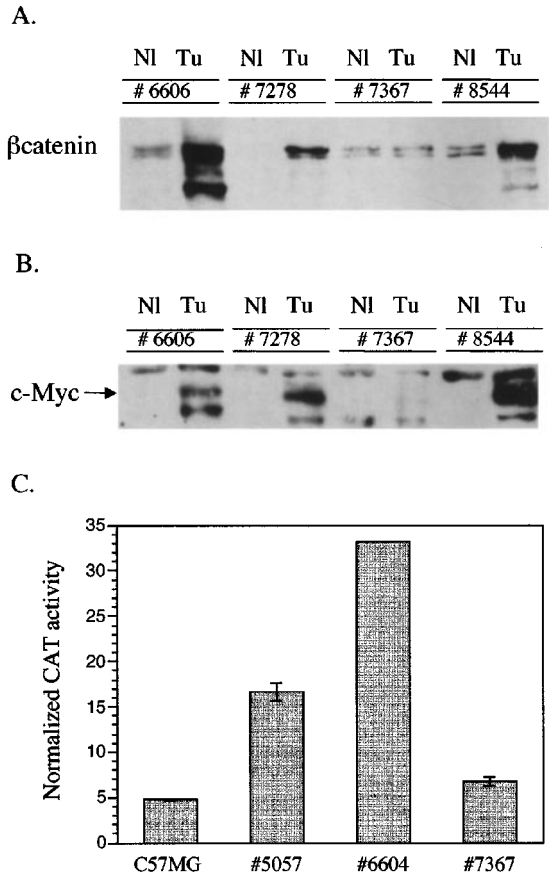

Figure 8 Expression and activity of β-catenin, NF-κB and c-*myc* in transgenic female MMTV-CK2α breast tumors. Forty micrograms of nuclear extracts obtained from paired normal mammary glands (Nl) and breast tumors (Tu) of transgenic mice were subjected to Western blotting with β-catenin (**a**) and c-Myc antibodies (**b**). Note the increase in β-catenin and c-*myc* levels in three out of four breast tumors. Equal loading was verified with Ponceau S staining. (**c**) Transgenic breast tumor and C57MG cell lines were transfected with an NF-κB-CAT reporter construct and mutant control to evaluate NF-κB transcriptional activity. Transfection efficiency was normalized using an SV40βgal reporter construct; data is expressed as mean and standard deviation of duplicate transfections. The increase in NF-κB transcriptional activity in the tumor cell lines averages fourfold over the control cell line

We and others have demonstrated constitutive high levels of NF-κB in rat and human breast cancer (Foo and Nolan, 1999; Kim *et al*., 2000; Nakshatri *et al*., 1997; Romieu-Mourez *et al*., 2001; Sovak *et al*., 1997). To determine whether this is also the case in the CK2α transgenic mammary tumors, we first examined nuclear NF-κB in primary CK2α transgenic tumors. While all four specimens examined contained constitutive nuclear NF-κB (data not shown), some of the specimens derived from the primary tumors also proved to be contaminated with lymphocytes. Thus, to determine whether there is transcriptionally active NF-κB in the malignant mammary epithelial cells, three cell lines derived from the primary tumors were transfected *in vitro* with an NF-κB-dependent chloramphenicol acetyl transferase (CAT) reporter. As a control, we used C57MG normal mouse mammary epithelial cell line (Howard *et al*., 1983). To normalize for transfection efficiency, CMV-βgal was transfected in parallel. All three cell lines exhibited higher CAT activity than the C57MG control cell line, with an average fourfold activation, indicating that the transgenic tumor lines contain transcriptionally active NF-κB (Figure 8c).

Overexpression of the c-*myc* oncogene (Seldin and Leder, 1995) or loss of *p53* (Landesman-Bollag *et al*., 1998) are collaborating events with CK2α in lymphomagenesis, and we hypothesized c-*myc* overexpression or *p53* loss might occur in CK2α transgenic mammary tumorigenesis. In particular, we expected to find elevated c-Myc expression, as c-*myc* is reported to be a transcriptional target of both NF-κB and β-catenin (Duyao *et al*., 1990; He *et al*., 1998; Kessler *et al*., 1992). Levels of c-Myc were determined by Western blot analysis of tumor tissue extracts. Three out of four tumors analysed had evidence of elevated c-Myc protein levels (Figure 8b). On the other hand, in the majority of primary tumors, *p53* mRNA was expressed and appeared normal (not shown). Thus, *p53* loss does not appear to be a frequent event in these transgenic mammary tumors.

Discussion

This study demonstrates a pathogenic relationship between protein kinase CK2 expression and mammary tumorigenesis. In carcinogen-induced rat mammary tumors and spontaneous human breast cancers, an association between CK2 levels and breast cancer was found; a transgenic mouse model demonstrated causality, as dysregulated expression of CK2α in the mammary gland promotes dysplasia and eventually cancer.

Carcinogen-induced mammary tumors produced in rats by administration of the polycyclic aromatic hydrocarbon (PAH) DMBA is a well-studied model system that has revealed molecular pathways of breast carcinogenesis (Kim *et al*., 2000; Ohi and Yoshida, 1992). This model is very relevant to human breast cancer because of the postulated role of environmental carcinogens in human mammary tumorigenesis (Morris and Seifter, 1992). A complex cellular metabolic response is initiated by the administration of DMBA, leading to DNA damage (Nebert *et al*., 1987; Randerath *et al*., 1985; Safe, 1984). Our data indicate that upregulation of CK2 protein and activity is also a component of this response, and suggest that there is a requirement for upregulation of this kinase in the development and/or maintenance of DMBA-induced mammary tumorigenesis. The relevance of CK2 to human mammary tumorigenesis is supported by our demonstration of elevated CK2 expression in human breast cancer specimens; at this time we cannot determine whether this occurs through the action of environmental pollutants or alternative molecular mechanisms.

These data are consistent with CK2 upregulation as either a consequence or a cause of mammary

gland transformation. To test the hypothesis that the overexpression of CK2 may directly contribute to mammary tumorigenesis, we used the MMTV-LTR to misexpress CK2α in the mammary epithelium of transgenic mice. Females from four independent transgenic lines exhibited histological abnormalities of the mammary gland, such as defects in involution and dysplasia. Several laboratories have observed incomplete involution in multiparous wild type FVB/N mice (Nieto *et al*., submitted); thus, this phenotype in our mice may be related to a background strain effect rather than to an effect of transgene expression. However, the dysplasic lesions observed in our transgenics are uncommon in wild type FVB/N mice and can be attributed to transgene expression; such lesions are thought to be precancerous (Cardiff and Wellings, 1999). Thirty per cent of the female transgenics developed mammary adenocarcinomas. In FVB/N mice these are rare as spontaneous malignancies (Mahler *et al*., 1996; RD Cardiff, unpublished observation). Furthermore, the bulk of the histological subtypes in our transgenics are glandular and papillary adenocarcinomas, and spindle cell carcinomas, while the rare spontaneous tumors in FVB/N are ketatoacanthomas or squamous cell carcinomas. This phenotypic difference implies a transgene-specific effect in the formation of MMTV-CK breast tumors.

The tumors had very high levels of CK2 protein and activity, suggesting that CK2 activity was required for ongoing tumor growth. This is supported by experiments with a selective pharmacologic inhibitor of CK2, the flavonoid apigenin, which inhibits growth of the CK2α transgenic breast cancer cell lines *in vitro* (not shown). Upregulation of the CK2α transgenic mRNA was only detected in some tumors (not shown); thus, we presume that the CK2 protein is derived from both transgene and endogenous CK2 expression. Variable transgene expression in tumors has been reported by other investigators and has been attributed in some cases to a hit and run mechanism in which the transgene mediates early transformation events but is not required in later steps of the process. EMT occurring in some of our transgenics could be another factor accounting for downregulation of transgene expression; as MMTV-LTR expression is restricted to epithelial and lymphoid cells, we would not expect mesenchymal tissue to express the transgene. Thus we predict that transgene expression would be shut off in our transgenic spindle cell carcinomas, since they have undergone EMT.

The variety of histologic patterns of the transgenic tumors is consistent with a variable and presumably stochastic activation of cooperating or downstream molecular pathways. The requirement for multiple additional steps to mammary transformation was also suggested by the long latency of tumor onset. We screened for such events based upon prior data about CK2-dependent pathways in growth control and cancer. CK2 participates in Wnt/β-catenin signaling (Song *et al*., 2000) and in the regulation of IκB turnover and NF-κB activity in mammary epithelial cells (Romieu-Mourez *et al*., 2001). In the CK2α transgenic breast tumors, upregulation of the Wnt/β-catenin pathway and of NF-κB occurred frequently. The role of the Wnt/β-catenin pathway is well-established in mouse mammary tumors (Barker *et al*., 1999; Bui *et al*., 1997; Dale *et al*., 1996; Huguet *et al*., 1994; Tsukamoto *et al*., 1988), and in humans it is emerging as an increasingly important pathway to breast cancer (Schlosshauer *et al*., 2000; Kuhl *et al*., 2000). CK2 phosphorylates the dishevelled signaling intermediates (Song *et al*., 2000; Willert *et al*., 1997) and β-catenin itself (Song *et al*., 2000). The NF-κB pathway has recently been documented to also play an important role in early mammary tumorigenesis (Kim *et al*., 2000; Sovak *et al*., 1997). Signal-dependent activation of NF-κB occurs in the cytoplasm via phosphorylation and degradation of IκBα which free the NF-κB subunits, allowing them to translocate to the nucleus and be transcriptionally active (reviewed in Israel, 2000). CK2 phosphorylates the C-terminal PEST domain of IκBα constitutively, and this has been found to also play a significant role in regulating IκB turnover, particularly with respect to regulation of basal levels of nuclear NF-κB (Bren *et al*., 2000; Heilker *et al*., 1999; Janosch *et al*., 1996; Lin *et al*., 1996; McElhinny *et al*., 1996; Pando and Verma, 2000; Schwarz *et al*., 1996; Tran *et al*., 1997). CK2 phosphorylates the p65 subunit of NF-κB directly (Bird *et al*., 1997). The transgenic CK2α mammary tumors have active nuclear NF-κB, suggesting cooperation between CK2 and NF-κB in mammary tumorigenesis.

c-*myc* is an important oncogene regulated by both Wnt/β-catenin signaling and by NF-κB (Duyao *et al*., 1990; He *et al*., 1998; Kessler *et al*., 1992). Bitransgenic expression of CK2α and c-*myc* in lymphocytes has demonstrated the potent transforming potential of co-expression of these genes (Seldin and Leder, 1995). c-Myc was well-expressed in the CK2α mammary tumors. On the other hand, although CK2α and *p53* loss are also collaborative in lymphoid transformation, we found evidence of *p53* loss in only two of the transgenic mammary tumors (data not shown).

In summary, we find increased expression and activity of protein kinase CK2 in breast tumors of human and rodent origin. Enforced overexpression of CK2α in the mammary gland of transgenic mice substantiates a model in which dysregulation of CK2 promotes hyperplasia and neoplasia of the mammary gland, although the latency of tumor formation points to a multistep pathway of tumorigenesis. Secondary events that may synergize with CK2 in mammary tumorigenesis include activation of the NF-κB and Wnt/β-catenin pathways, with c-*myc* as critical downstream transcription factor target. CK2 inhibitors may have a potential role in the treatment or prevention of mammary tumorigenesis.

Materials and methods

Human and rat tissues

Breast tumors were induced in rats by a single gavage with 15 mg/kg of DMBA at 8 weeks of age. The rats were sacrificed 17 weeks later. Tumors and pooled normal mammary glands were snap frozen at the time of necropsy; portions of these tissues were used for other experiments on NF-κB and breast cancer (Kim *et al*., 2000). All animal experimentation was carried out in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care and with approval of the Boston University School of Medicine I.A.C.U.C. committee. Unneeded portions of anonymous frozen human breast tissue samples and breast tumor specimens were obtained with approval of the Institutional Review Board, Boston Medical Center.

Transgenic animals

Mice from the FVB/N strain were utilized to generate transgenic animals. The murine CK2α catalytic subunit cDNA was subcloned into a vector in which the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) directs expression chiefly to the mammary epithelium, with ras 5′ untranslated sequences provided upstream of the cDNA and an SV40 intron and polyadenylation signal downstream (Sinn *et al*., 1987). Plasmid sequences were removed by restriction digestion at the *Sal*I and *Spe*I sites, and the excised transgene construct was gel purified and microinjected into pronuclei of fertilized one-cell zygotes. There were reimplanted into pseudopregnant foster mothers and the offspring were screened for presence of the transgene. Carriers were bred to establish four independent transgenic lines. Female transgenic mice were continuously bred to induce transgene expression through activation of the hormone-dependent MMTV-LTR. Mice were monitored weekly for the appearance of tumors. Ill mice were sacrificed and tissues collected for histopathological analysis, cell culture, RNA and protein analyses. To assess expression levels of the transgene in the mouse organs, tissues were collected from 6–8 week-old virgin females, from females that were pregnant, and from females at day 7 of mammary gland involution. Mice were housed in a 2-way barrier at the Boston University School of Medicine Core Transgenic Mouse Facility in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care.

Genotyping and expression analyses

Genomic DNA was extracted from 1 cm tail tip samples by digestion with proteinase K, high salt extraction of proteins, and ethanol precipitation of DNA. Genotyping was performed by Southern blot analysis or by PCR. For Southern analysis, DNA was digested with *Bam*HI, electrophoresed on 1% agarose gels (FMC Rockland, ME, USA), capillary blotted with 0.4 N NaOH onto nylon membranes (Gene Screen Plus, NEN, Boston, MA, USA), and hybridized with a α-^{32}P-radiolabeled 1.1 Kb *Bam*HI fragment comprising the entire mouse CK2α cDNA (Seldin and Leder, 1995). For PCR, a 290 bp fragment of the transgene was amplified using a sense oligonucleotide primer from the mouse CK2α cDNA coding sequence (5′-GGGATTTCTTCAGTGCCATC-3′) and an antisense oligonucleotide from the SV40 poly(A)$^+$ signal of the vector construct (5′-CCCATTCATAAGTTCCATAG-3′). PCR was performed in a thermal cycler (MJ Research, Watertown, MA, USA) by denaturing at 95°C for 30 s, annealing at 52°C for 40 s, and extending at 72°C for 40 s for 30 cycles.

For expression analysis, total RNA was extracted from mouse tissues (Chirgwin *et al*., 1979). After DNase treatment (Roche, Indianapolis, IN, USA), RNA was re-extracted and ethanol precipitated. Five to 10 micrograms of RNA were then reverse transcribed using the ProSTAR™ First Strand RT–PCR kit (Stratagene, La Jolla, CA, USA). PCR was performed with CK2α and SV40 primers described above, for 35 cycles, to detect the transgene mRNA. Both spliced and unspliced transgene mRNA could be amplified, as there is a splice donor and acceptor in the amplified region of the SV40 polyA tail. The quality of first strand synthesis was verified with *hprt* amplification. PCR was performed by denaturing at 95°C for 30 s, annealing at 55°C for 40 s, and extending at 72°C for 40 s for 30 cycles using the sense oligonucleotide (5′-GTTGGATACAGGCCAGACTTTGT-TG-3′) and the antisense oligonucleotide (5′-GAGGGTAGGCTGGCCTA-TAGGCT-3′) (Johnson *et al*., 1988).

Western blot analyses

Protein extracts were prepared by homogenizing frozen tumors or mammary gland specimens in lysis buffer containing a cocktail of protease and phosphatase inhibitors (40 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 125 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM Na_3VO_4, and 10 mM sodium pyrophosphate) followed by gentle centrifugation to remove debris. The same method was used for whole tissue culture cell extracts, after directly lysing the cells in lysis buffer. Nuclear extracts from breast tissue samples or mammary tumor cell lines were prepared as described (Sovak *et al*., 1997). Lysate protein content was quantified by BCA protein assay (Pierce, Rockford, IL, USA). Samples (40–100 μg) were electrophoresed on 7.5% or 10% SDS polyacrylamide gels (Laemmli, 1970) and transferred onto nitrocellulose filters (Schleicher and Schuell, Keene, NH, USA). Filters were blocked in 5% milk, 1 × PBS, 0.05% Tween, incubated with primary antibody, washed, incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed again and visualized by ECL (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Primary antibodies were rabbit α-human-CK2α (Stressgen, Victoria, BC), rabbit α-CK2β (a generous gift from N Chester and D Marshak), MAb anti-β-catenin (Signal Transduction, Lexington, KY, USA), and rabbit anti-c-myc (UBI). Densitometry analysis was performed using Quantity One software (BioRad, Hercules, CA, USA). Verification of equal loading was done by staining the nitrocellulose filters with Ponceau S (Sigma, St Louis, MO, USA). This was found to be more accurate than quantitation of selected cytoskeletal proteins such as actin or tubulin or of cytokeratins, since the tumors clearly exhibited variable expression of these proteins than did not correlate with the non-selective BCA or Ponceau S techniques, as has been reported by others (Adam *et al*., 1998; Perfetti *et al*., 1991). For immunofluorescence, we used the broad spectrum anti-keratin antibody, clone C-11, (NeoMarkers, Union City, CA, USA).

Immunohistochemistry

The tumor samples were sectioned and placed on glass slides. They were analysed using immunohistochemical stains for

vimentin (PH 514, diluted 1 : 2000, Binding Site, San Diego, CA, USA), smooth muscle actin (A2547 diluted 1 : 1000, Sigma), cytokeratin 8 (PH 192 diluted 1 : 200, Binding Site San Diego, CA, USA) and cytokeratin 14 (PH 503 diluted 1 : 200, Binding Site). The anti-smooth muscle actin, a mouse monoclonal antibody, was amplified and detected using the 'mouse on mouse' kit K3954 (Dako Corp., Carpinteria, CA, USA). The other antibodies, all sheep anti-peptide polyclonal antibodies (Binding Site), were detected and amplified using biotinylated rabbit anti-sheep IgG, diluted 1 : 1000 (BA 6000 Vector Labs, Burlingame, CA, USA). Microwaving of the sections in citrate buffer was used for antigen retrieval. The general conditions and times were according to manufacturer's instructions. However, all antibodies and detection systems were optimized using a prepared panel of mouse control tissues. Positive and negative controls were included with each experimental run as quality controls. The sections were lightly counter-stained with hematoxylin to provide contrast. Each slide contained sufficient normal tissue to provide internal controls for the observer. Digital images were captured using a Kontron digital camera with ProgRes software and processed in PhotoShop.

Histology

Upon necropsy, tumors and organs were removed and immediately fixed in Optimal Fix (American Histology Reagent Company, Inc) and shipped in alcohol. The tissues were processed, embedded in paraffin and sectioned at 7 microns. The sections were mounted on glass slides and stained with hematoxylin and eosin using routine laboratory procedures in the Transgenic Core Pathology Laboratory at the University of California, Davis. Sections were compared with other specimens in the extensive mouse mammary tumor database (http://www-mp.ucdavis.edu/tgmice/firststop.html).

Cell culture

Transgenic breast tumor cell lines were generated by mincing the tumor in DMEM under sterile conditions followed by culture in DMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin (Mediatech Inc. Cellgro, Herndon, VA, USA) in a 5% CO_2 incubator at 37°C to establish immortalized cell lines. In some cases, the tumorigenic nature of these cell lines was confirmed by transplantation of approximately 10^6 cells subcutaneously into syngeneic recipients.

CK2 peptide kinase assay

Samples (2–5 μg) of protein lysate were incubated in a 1 mM solution of the specific protein kinase CK2 substrate peptide RRREEETEEE (Sigma-Genosys, The Woodlands, TX, USA) in CK2 kinase buffer (100 mM Tris pH 8.0, 20 mM $MgCl_2$, 100 mM NaCl, 50 mM KCl, 0.1 μg/μl BSA and 100 μM Na_3VO_4) and 5 μCi of [γ-^{32}P]GTP (6000 Ci/mmol) at 30°C for 10 min (Kuenzel *et al*., 1987). The reaction was stopped by adding 25 μl of 100 mM ATP in 0.4 N HCl. Samples were spotted onto a P81 Whatman filter and washed in 150 mM H_3PO_4, four times, 5 min each, to remove unincorporated [γ-^{32}P]GTP. Phosphorylated peptides were quantified in an automatic scintillation counter. The samples were assayed in duplicate and background kinase activity in the absence of the specific peptide substrate was subtracted. The statistical significance of differences in activity between pairs was determined by the *t*-test for Paired Two Sample for Means.

NF-κB reporter assay

To evaluate NF-κB transcriptional activity, wild type (E8-CAT) and mutant (dmE8-CAT) NF-κB element-thymidine kinase (TK) promoter-CAT reporter vectors, containing two copies of either the wild type or mutant NF-κB element from upstream of the c-*myc* promoter were employed (Duyao *et al*., 1990). Cells were transfected, in duplicate, using Fugene reagent (Boehringer Mannheim) according to the manufacturer's directions. CAT assays were performed, and the pSVβ-galactosidase (SV40βgal) reporter vector was used to normalize transfection efficiency, as previously described (Arsura *et al*., 1997). The mean and standard deviation of duplicates were calculated.

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References

Adam L, Vadlamudi R, Kondapaka SB, Chernoff J, Mendelsohn J and Kumar R. (1998). *J. Biol. Chem.*, **273,** 28238–28246.

Ahmed K. (1994). *Cell. Mol. Biol. Res.*, **40,** 1–11.

Allende JE and Allende CC. (1995). *FASEB J.*, **9,** 313–323.

Arsura M, FitzGerald MJ, Fausto N and Sonenshein GE. (1997). *Cell Growth Differ.*, **8,** 1049–1059.

Barker N, Huls G, Korinek V and Clevers H. (1999). *Am. J. Pathol.*, **154,** 29–35.

Birchmeier C, Birchmeier W and Brand-Saberi B. (1996). *Acta. Anat.*, **156,** 217–226.

Bird TA, Schooley K, Dower SK, Hagen H and Virca GD. (1997). *J. Biol. Chem.*, **272,** 32606–32612.

Bren GD, Pennington KN and Paya CV. (2000). *J. Mol. Biol.*, **297,** 1245–1258.

Bui TD, Rankin J, Smith K, Huguet EL, Ruben S, Strachan T, Harris AL and Lindsay S. (1997). *Oncogene,* **14,** 1249–1253.

Cardiff RD. (1996). *J. Mammary Gland Biol. Neoplasia,* **1,** 61–73.
Cardiff RD and Wellings SR. (1999). *J. Mammary Gland Biol. Neoplasia,* **4,** 105–122.
Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ. (1979). *Biochemistry,* **18,** 5294–5299.
Dale TC, Weber-Hall SJ, Smith K, Huguet EL, Jayatilake H, Gusterson BA, Shuttleworth G, O'Hare M and Harris AL. (1996). *Cancer Res.,* **56,** 4320–4323.
Daya-Makin M, Sanghera JS, Mogentale TL, Lipp M, Parchomchuk J, Hogg JC and Pelech SL. (1994). *Cancer Res.,* **54,** 2262–2268.
Duyao MP, Buckler AJ and Sonenshein GE. (1990). *Proc. Natl. Acad. Sci. USA,* **87,** 4727–4731.
Faust RA, Gapany M, Tristani P, Davis A, Adams GL and Ahmed K. (1996). *Cancer Lett.,* **101,** 31–35.
Foo SY and Nolan GP. (1999). *Trends Genet.,* **15,** 229–235.
Ford HL, Landesman-Bollag E, Dacwag CS, Stukenberg PT, Pardee AB and Seldin DC. (2000). *J. Biol. Chem.,* **275,** 22245–22254.
Gapany M, Faust RA, Tawfic S, Davis A, Adams GL and Ahmed K. (1995). *Mol. Med.,* **1,** 659–666.
Glover III CV. (1998). *Prog. Nucleic. Acid. Res. Mol. Biol.,* **59,** 95–133.
Guerra B and Issinger OG. (1999). *Electrophoresis,* **20,** 391–408.
He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B and Kinzler KW. (1998). *Science,* **281,** 1509–1512.
Heilker R, Freuler F, Pulfer R, Di Padova F and Eder J. (1999). *Eur. J. Biochem.,* **259,** 253–261.
Heller-Harrison RA, Meisner H and Czech MP. (1989). *Biochemistry,* **28,** 9053–9058.
Howard DK, Schlom J and Fischer PB. (1983). *In Vitro,* **19,** 58–66.
Huggins C, Grand LC and Brillantes FP. (1961). *Nature,* **189,** 204–207.
Huguet EL, McMahon JA, McMahon AP, Bicknell R and Harris AL. (1994). *Cancer Res.,* **54,** 2615–2621.
Israel A. (2000). *Trends Cell Biol.,* **10,** 129–133.
Issinger OG. (1993). *Pharmacol. Ther.,* **59,** 1–30.
Janosch P, Schellerer M, Seitz T, Reim P, Eulitz M, Brielmeier M, Kolch W, Sedivy JM and Mischak H. (1996). *J. Biol. Chem.,* **271,** 13868–13874.
Johnson GG, Kronert WA, Bernstein SI, Chapman VM and Smith KD. (1988). *J. Biol. Chem.,* **263,** 9079–9082.
Kelliher MA, Seldin DC and Leder P. (1996). *EMBO J.,* **15,** 5160–5166.
Kessler DJ, Duyao MP, Spicer DB and Sonenshein GE. (1992). *J. Exp. Med.,* **176,** 787–792.
Kim DW, Sovak MA, Zanieski G, Nonet G, Romieu-Mourez R, Lau AW, Hafer LJ, Yaswen P, Stampfer M, Rogers AE, Russo J and Sonenshein GE. (2000). *Carcinogenesis,* **21,** 871–879.
Kuenzel EA, Mulligan JA, Sommercorn J and Krebs EG. (1987). *J. Biol. Chem.,* **262,** 9136–9140.
Kuhl M, Sheldahl LC, Park M, Miller JR and Moon RT. (2000). *Trends Genet.,* **16,** 279–283.
Laemmli UK. (1970). *Nature,* **227,** 680–685.
Landesman-Bollag E, Channavajhala PL, Cardiff RD and Seldin DC. (1998). *Oncogene,* **16,** 2965–2974.
Lin R, Beauparlant P, Makris C, Meloche S and Hiscott J. (1996). *Mol. Cell Biol.,* **16,** 1401–1409.
Liu ZP, Galindo RL and Wasserman SA. (1997). *Genes Dev.,* **11,** 3413–3422.
Mahler JF, Stokes W, Mann PC, Takaoka M and Maronpot RR. (1996). *Toxicol. Pathol.,* **24,** 710–716.
McElhinny JA, Trushin SA, Bren GD, Chester N and Paya CV. (1996). *Mol. Cell. Biol.,* **16,** 899–906.
Mestres P, Boldyreff B, Ebensperger C, Hameister H and Issinger OG. (1994). *Acta. Anat.,* **149,** 13–20.
Mitev V, Miteva L, Botev I and Houdebine LM. (1994). *J. Dermatol. Sci.,* **8,** 45–49.
Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B and Kinzler KW. (1997). *Science,* **275,** 1787–1790.
Morris JJ and Seifter E. (1992). *Med. Hypotheses,* **38,** 177–184.
Munstermann U, Fritz G, Seitz G, Lu YP, Schneider HR and Issinger OG. (1990). *Eur. J. Biochem.,* **189,** 251–257.
Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet Jr RJ and Sledge Jr GW. (1997). *Mol. Cell. Biol.,* **17,** 3629–3639.
Nebert DW, Jaiswal AK, Meyer UA and Gonzalez FJ. (1987). *Biochem. Soc. Trans.,* **15,** 586–589.
Nusse R and Varmus HE. (1982). *Cell,* **31,** 99–109.
Ohi Y and Yoshida H. (1992). *Virchows Arch. B Cell. Pathol. Inc. Mol. Pathol.,* **62,** 365–370.
ole-MoiYoi OK, Brown WC, Iams KP, Nayar A, Tsukamoto T and Macklin MD. (1993). *EMBO J.,* **12,** 1621–1631.
Orlandini M, Semplici F, Ferruzzi R, Meggio F, Pinna LA and Oliviero S. (1998). *J. Biol. Chem.,* **273,** 21291–21297.
Pancetti F, Bosser R, Itarte E and Bachs O. (1996). *Biochem. Biophys. Res. Commun.,* **218,** 35–39.
Pando MP and Verma IM. (2000). *J. Biol. Chem.,* **275,** 21278–21286.
Pena JM, Itarte E, Domingo A and Cusso R. (1983). *Cancer Res.,* **43,** 1172–1175.
Pepperkok R, Lorenz P, Ansorge W and Pyerin W. (1994). *J. Biol. Chem.,* **269,** 6986–6991.
Perez M, Grande J and Itarte E. (1988). *FEBS Lett.,* **238,** 273–276.
Perfetti V, Manenti G and Dragani TA. (1991). *Leukemia,* **5,** 1110–1112.
Pinna LA and Meggio F. (1997). *Prog. Cell Cycle Res.,* **3,** 77–97.
Polakis P. (1999). *Curr. Opin. Genet. Dev.,* **9,** 15–21.
Randerath E, Agrawal HP, Weaver JA, Bordelon CB and Randerath K. (1985). *Carcinogenesis,* **6,** 1117–1126.
Rogers AE and Wetsel WC. (1981). *Cancer. Res.,* **41,** 3735–3737.
Roig J, Krehan A, Colomer D, Pyerin W, Itarte E and Plana M. (1999). *Mol. Cell. Biochem.,* **191,** 229–234.
Romieu-Mourez R, Landesman-Bollag E, Seldin DC, Traish AM, Mercurio F and Sonenshein GE. (2001). *Cancer Res.,* (in press).
Roose J and Clevers H. (1999). *Biochim. Biophys. Acta,* **1424,** M23–M37.
Safe S. (1984). *Crit. Rev. Toxicol.,* **13,** 319–395.
Schlosshauer PW, Brown SA, Eisinger K, Yan Q, Guglielminetti ER, Parsons R, Ellenson LH and Kitajewski J. (2000). *Carcinogenesis,* **21,** 1453–1456.
Schwarz EM, Van AD and Verma IM. (1996). *Mol. Cell. Biol.,* **16,** 3554–3559.
Seldin DC and Leder P. (1995). *Science,* **267,** 894–897.
Sinn E, Muller W, Pattengale P, Tepler I, Wallace R and Leder P. (1987). *Cell,* **49,** 465–475.
Song DH, Sussman DJ and Seldin DC. (2000). *J. Biol. Chem.,* **275,** 23790–23797.
Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM and Sonenshein GE. (1997). *J. Clin. Invest.,* **100,** 2952–2960.
Stewart TA, Pattengale PK and Leder P. (1984). *Cell,* **38,** 627–637.

Toczyski DP, Galgoczy DJ and Hartwell LH. (1997). *Cell,* **90,** 1097 – 1106.
Tran K, Merika M and Thanos D. (1997). *Mol. Cell. Biol.,* **17,** 5386 – 5399.
Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T and Varmus HE. (1988). *Cell,* **55,** 619 – 625.
Webster MA, Hutchinson JN, Rauh MJ, Muthuswamy SK, Anton M, Tortorice CG, Cardiff RD, Graham FL, Hassell JA and Muller WJ. (1998). *Mol. Cell. Biol.,* **18,** 2344 – 2359.
Willert K, Brink M, Wodarz A, Varmus H and Nusse R. (1997). *EMBO J.,* **16,** 3089 – 3096.
Xu, X, Toselli PA, Russell LD and Seldin Dc. (1999). *Nat. Genet.,* **23,** 118 – 121.

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Roles of IKK Kinases and Protein Kinase CK2 in Activation of Nuclear Factor-κB in Breast Cancer[1]

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ABSTRACT

Nuclear factor-κB (NF-κB)/Rel transcription factors regulate genes that control cell proliferation, survival, and transformation. In normal breast epithelial cells, NF-κB/Rel proteins are mainly sequestered in the cytoplasm bound to one of the specific inhibitory IκB proteins, whereas in breast cancers they are activated aberrantly. Human breast tumor cell lines, carcinogen-transformed mammary epithelial cells, and the majority of primary human or rodent breast tumor tissue samples express constitutively high levels of nuclear NF-κB/Rel. To begin to understand the mechanism of this aberrant NF-κB/Rel expression, in this study we measured the activity of the major kinases implicated in regulation of IκB stability, namely IKKα, IKKβ, and protein kinase, CK2 (formerly casein kinase II). Hs578T, D3-1, and BP-1 breast cancer cell lines displayed higher levels of IKKα, IKKβ, and CK2 activity than untransformed MCF-10F mammary epithelial cells. Inhibition of IKK activity upon expression of dominant negative kinases or of CK2 activity by treatment with selective inhibitors decreased NF-κB/Rel activity in breast cancer cells. Inactivation of the IκB kinase complex in Hs578T cells via expression of a dominant negative IKKγ/NF-κB essential modulator/IKK-associated protein 1 reduced soft agar colony growth. Thus, the aberrant expression of CK2 or IKK kinases promotes increased nuclear levels of NF-κB/Rel and transformation of breast cancer cells. Furthermore, primary human breast cancer specimens that displayed aberrant constitutive expression of NF-κB/Rel were found to exhibit increased CK2 and/or IKK kinase activity. These observations suggest these kinases play a similar role in an intracellular signaling pathway that leads to the elevated NF-κB/Rel levels seen in primary human mammary tumors and, therefore, represent potential therapeutic targets in the treatment of patients with breast cancer.

INTRODUCTION

NF-κB[3]/Rel is a family of dimeric transcription factors distinguished by the presence of a 300-amino acid region, termed the Rel homology region, which determines much of its function (1). Classical NF-κB is a heterodimer composed of a RelA (p65) and p50 subunit. In most cells, other than B lymphocytes, NF-κB/Rel proteins are sequestered in the cytoplasm bound to the specific IκB inhibitory proteins, of which IκB-α is the paradigm. Although the v-*rel* gene, carried by the highly oncogenic avian reticuloendotheliosis virus strain T, is able to cause tumors in birds, the role of NF-κB in mammalian cancers was less clear for many years (2). Several oncogenic mammalian viruses were shown to activate NF-κB. For example, the product of the *tax* gene of the HTLV-1 virus activates NF-κB (3), which we showed mediates transactivation of the c-*myc* promoter (4). Recently, we and others have demonstrated a role for NF-κB/Rel factors in breast cancer (5, 6). High levels of nuclear NF-κB/Rel were found in human breast tumor cell lines, carcinogen-transformed mammary epithelial cells, and the majority of primary human or rodent breast tumor tissue samples. In contrast, untransformed breast epithelial cells and normal rat mammary glands contained low basal levels (5, 6).

Several laboratories have found that increased NF-κB expression in tumor cells correlates with decreased stability of IκB proteins. For example, we recently showed that elevated levels of NF-κB in the D3-1 and BP-1 cell lines, derived by *in vitro* transformation of MCF-10F breast epithelial cells by 7,12-dimethylbenz(*a*)anthracene and benzo[a]pyrene, respectively, correlated with a decrease in the half-life of IκB-α protein (7). Much progress has been made in elucidating the kinases that regulate IκB-α stability. These appear to function via phosphorylation of NH_2-terminal or COOH-terminal sites of IκB-α. A variety of agents that induce NF-κB/Rel have been found to mediate activation of NF-κB via phosphorylation of IκB on two NH_2-terminal serine residues in a large multi-subunit complex (8). The IκB kinase complex consists of two IκB kinases, IKKα or IKK-1 and IKKβ or IKK-2 (8). In addition, there is a M_r 48,000 essential component, alternatively termed NEMO, IKKγ, or IKKAP1 (9–11). The IKKγ/NEMO/IKKAP1 protein is essential for function of the IκB kinase complex, and mutants can completely inhibit all of the IKK kinase function (9–11). The IKKα and IKKβ protein serine kinases contain a leucine zipper and a helix-loop-helix motif in the COOH-terminal region and a kinase domain in the NH_2-terminal region (8). Activation of the IκB kinase complex is mediated via phosphorylation of either IKKα or IKKβ (12, 13). IκB-α is then recruited into the IκB kinase complex, where it is phosphorylated by the functional IKKα/IKKβ heterodimer at serine residues at positions 32 and 36. The NH_2-terminal IKK phosphorylation sites have been recently shown to play a role in the signal-induced degradation of both free and NF-κB-bound forms of IκB-α (14). IκB-α phosphorylation is followed by ubiquitination and rapid degradation, allowing for migration of the released NF-κB to the nucleus (15).

In addition to the NH_2-terminal residues, it has been shown that phosphorylation of serines and/or threonines within the COOH-terminal PEST domain of IκB-α affects the stability of the protein (16–18). The kinase responsible for this phosphorylation has been identified as the serine/threonine protein kinase CK2 (CK2, formerly casein kinase II). CK2 phosphorylates IκB-α preferentially at Ser-283, Ser-288, T-291, and Ser-293 within the PEST domain (18, 19). CK2 is a ubiquitously expressed and constitutively active kinase that exists in cells as a heterotetrameric protein containing two catalytic (α/α, α/α′, or α′/α′) and two regulatory (β/β) subunits (20, 21). CK2-mediated phosphorylation of IκB-α in the PEST domain has been implicated in the basal and signal-dependent turnover of free and NF-κB-bound IκB-α (14, 16, 18, 19). The mechanisms of the basal degradation of IκB-α are not fully understood, although it has been suggested that it involves IκB complex

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[3] The abbreviations used are: NF-κB, nuclear factor-κB; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; EMSA, electric mobility shift analysis; GST, glutathione *S*-transferase; IKKAP1, IKK-associated protein 1; NEMO, NF-κB essential modulator; PMSF, phenylmethylsulfonyl fluoride; WCE, whole cell extract; PNPP, p-nitrophenyl phosphate; wt, wild-type; TNF, tumor necrosis factor; ER, estrogen receptor; PR, progesterone receptor; HTLV, T-cell leukemia virus; β-gal, β-galactosidase.

phosphorylation, ubiquitination, and degradation by the 26S proteasome (14) or, alternatively, a calpain-mediated mechanism (22). These findings have implicated CK2 in control of intrinsic IκB-α stability and, thereby, in constitutive activation of NF-κB, although this remains to be proven directly.

To begin to evaluate the mechanism of aberrant activation of NF-κB in breast cancer, in this study we have assessed the activity of the IKKα and IKKβ components of the IκB kinase complex and of CK2 in human breast cancer cell lines and primary breast cancer specimens. We report that breast cancer cells in culture display elevated IKK and CK2 kinase activity. Inhibition of these activities reduces NF-κB activity. Furthermore, multiple primary breast cancer specimens that display aberrant constitutive expression of NF-κB exhibit increases in either IKK or CK2 kinase activity. We conclude that aberrant activation of IKK or CK2 leads to elevated nuclear NF-κB activity, which in turn can result in enhanced survival and transformed phenotype of breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions. The Hs578T tumor cell line, which was derived from a carcinosarcoma and is epithelial in origin, was grown as described previously (5). Where indicated, cells were incubated with 20–100 μM apigenin or 1–25 μg/ml emodin (both from Sigma Chemical Co.) dissolved in DMSO or similar dilution of DMSO as control. MCF-10F is a human mammary epithelial cell line, established from a patient with fibrocystic disease, which does not display malignant characteristics (23). The D3-1 and BP-1 lines were derived by 7,12-dimethylbenz(*a*)anthracene-mediated and benzo[a]pyrene-mediated transformation of MCF-10F cells, respectively, and were cultured as published previously (23).

Transfection Conditions. For transient transfection, cells were incubated for 12 to 16 h at 37°C with a solution of DNA and Fugene reagent according to the manufacturer's directions (Boehringer Mannheim). To evaluate transcriptional activity, cells were transfected in duplicate with wt (E8-CAT) and mutant (dmE8-CAT) NF-κB element-thymidine kinase promoter-CAT reporter vectors, containing two copies of either the wt or mutant NF-κB element from upstream of the c-*myc* promoter (4). CAT assays were performed as described previously (5). Alternatively, cells were transfected in triplicate with NF-κB element-luciferase reporter vector, driven by 3 NF-κB elements from upstream of the MHC class I promoter**,** kindly provided by Dr. A. Chan (Mt. Sinai School of Medicine, New York, NY; Ref. 24). Luciferase assays were performed as described previously (25). The CMV promoter β-gal reporter vector pON407, in which the five putative NF-κB sites within the CMV promoter have been removed (26), was used to normalize transfection efficiency as described previously (27). SD was obtained using the Student *t* test. The pRC-βactin-IKKαSS/AA vector, which expresses a phosphorylation-defective mutant IKKαSS/AA that functions as a dominant negative version of IKKα, and the parental pRC-βactin vector were as described (13). The pRC-βactin-IKKαSS/AA vector insert was subcloned into the pcDNA3 vector yielding pcDNA3-IKKαSS/AA. The plasmids pCMV-IKKβSS/AA and pCMV-IKKβSS/EE, allowing expression of a dominant negative mutant flag-tagged IKKβ, and a constitutively active flag-tagged IKKβ, respectively, have been described previously (13). The vector directing expression of dominant negative IKKγ/NEMO/IKKAP1 (9) was kindly provided by Drs. D. Rothwarf and M. Karin (University of California San Diego, La Jolla, CA).

Human Breast Cancer Specimen Analysis. Primary human breast cancer tissue specimens were obtained from patients undergoing surgery for treatment of breast cancer with approval of the Institutional Review Board of Boston Medical Center. Tumors were processed for steroid receptor analysis, and any remaining tissue was considered discarded material and used for subsequent analysis of NF-κB, and IKK and CK2 kinases. Tissues were stored frozen at −75°C until samples were processed for nuclear and cytoplasmic protein fractionation. Samples were pulverized on dry ice using a Bessman Tissue Pulverizer (Spectrum). Frozen tissue powder was homogenized (0.5 g/ml) in TEGT/MO buffer [50 mM Tris/HCl, 1 mM EDTA, 10% (v/v) glycerol, 10 mM monothioglycerol, and 10 mM sodium molybdate (pH 7.4) containing 0.02% sodium azide] using a Polytron. After the initial burst, proteolytic inhibitors were added to a final concentration as follows: 0.5 mM PMSF, 1 μg/ml leupeptin, 100 μg/ml aprotinin, 10 μg/ml pepstatin, and 100 μg/ml bacitracin. Homogenates were centrifuged for 10 min at 3000 rpm at 2°C to isolate crude nuclei. The nuclear pellet was washed 3× with buffer, and nuclear proteins were extracted as described previously (5). The supernatant was centrifuged at 100,000 × *g*, and the clear cytosolic extract was removed and stored frozen for analysis.

EMSA. The sequence of the wt URE NF-κB-containing oligonucleotide from the c-*myc* gene (4) is as follows: wt, 5′-GATCCAAGTCCGGGTTTTC-CCCAACC-3′. The core element is underlined. The mutant URE has two G to C-bp conversions, indicated in bold, which block NF-κB/Rel binding: 5′-GATCCAAGTCC**CC**TTTTCCCCAACC-3′. The sequence of the Sp1 oligonucleotide is 5′-ATTCGATCGGGGCGGGGCGACC-3′. The sequences of the PU.1- and TCF-1-containing oligonucleotides are as follows: PU.1, 5′-GATCTACTTCTGCTTTTG-3′; and TCF-1, 5′-GGGAGACTGAGAA-CAAAGCGCTCTCACAC-3′ (28). Nuclear extracts from breast tissue samples or breast cell lines were prepared, and samples (2.5–5 μg) were subjected to EMSA as described (5). For antibody supershift analysis, the binding reaction was first performed in the absence of the probe, the appropriate antibody was added, and the mixture was incubated for 16 h at 4°C. The probe was then added. The reaction was incubated an additional 30 min at 25°C, and the complexes resolved by gel electrophoresis, as above. Antibodies used included: anti-RelA subunit, sc-372; anti-p50 subunit, sc-114; anti-p52 subunit, sc-7386; and anti-c-Rel subunit, sc-71 (all of these were from Santa Cruz Biotechnology, Santa Cruz, CA). Where indicated, either 250 ng of IκB-α-GST fusion protein, GST alone, excess unlabeled wt, or mutant oligonucleotide was added to the binding reaction just before addition of the probe. Data were quantified by densitometry using a Molecular Dynamics densitometer.

Immunoblotting. Samples were separated by electrophoresis in polyacrylamide-SDS gels, transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA), and subjected to immunoblotting, as described previously (27). Antibodies specific for IKKα (M-280), IKKβ (H-470), and FLAG-pCruz Octa expression vector-encoded fusion proteins (D-8) were purchased from Santa Cruz Biotechnology. Phospho-IκB-α (Ser-32)-specific antibody was from New England Biolabs (Beverly, MA). The rabbit polyclonal antibody specific for the CK2α subunit of CK2 was from Stressgen (Victoria, British Columbia, Canada). A monoclonal antibody specific for β-actin (AC-15) was purchased from Sigma Chemical Co.

IKK Kinase Assay. To prepare WCEs, cells were washed with PBS, resuspended in cold kinase assay lysis buffer [20 mM Tris (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.25% NP40], and lysed by sonication. Debris was removed by centrifugation, and extracts precleared with protein A-Sepharose beads (Amersham Pharmacia Biotech AB) for 1 h at 4°C. The IKK complexes were isolated by immunoprecipitation from a 500-μl reaction mixture of PD buffer [40 mM Tris (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.1% NP40] containing 150 μg of cytoplasmic proteins and 1 μg of antibody against either IKKα (M-280), IKKβ (H-470), or Flag-tag (D-8). After washing, one-third of the immunoprecipitate was subjected to a kinase assay at 30°C for 45 min in kinase buffer C [20 mM HEPES (pH 7.7), 2 mM $MgCl_2$, 10 μM ATP, 3 μCi of [γ-^{32}P]ATP, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 μM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM DTT] containing 200 ng of wt GST-IκB-α fusion protein (GST-wtIκB-α) as substrate (13). Alternatively, a truncated (amino acid 1-54) mutant IκB-α-GST, in which Ser-32 and Ser-36 were replaced by alanines (A32,36mut GST-IκB-α), was used to assess kinase specificity. (Concentrations of the GST fusion protein preparations were monitored in Coomassie Blue-stained SDS-polyacrylamide gels by comparison to BSA standards.) The kinase reaction was stopped by the addition of 2 × SDS-PAGE sample buffer, subjected to SDS-PAGE analysis, and visualized by autoradiography. The remaining fraction was subjected to immunoblot analysis, as described above. SD was obtained using the Student *t* test.

CK2 Kinase Assay. For evaluation of IκB-phosphorylation directed by CK2, 10- to 20-μg WCEs prepared using kinase assay lysis buffer were diluted to 10-μl final volume with the same buffer. After the addition of 15 μl of buffer D [100 mM Tris (pH 8.0), 100 mM NaCl, 50 mM KCl, 20 mM $MgCl_2$, 100 μM Na_3VO_4, and 10 μCi of [γ-^{32}P]GTP], reactions were incubated at

30°C for 30 min in the presence of 200 ng GST-wtIκB-α as substrate. Alternatively, GST-Δ2IκB-α, with a deletion of amino acids 269–317 in the COOH-terminal PEST domain of IκB-α or GST-3CIκB-α with three point mutations at S283A, T291A, and T299A (17), kindly provided by Dr. J. Hiscott (Institut Lady Davis de Recherches Medicales, Montreal, Quebec, Canada), were used as substrates. Where indicated, 20–80 μM apigenin, 1–25 μg/ml emodin, or 0.58–1.46 mM CK2-specific peptide substrate RRREEETEEE (Sigma Genosys Inc.) was added to the kinase reaction. Alternatively, recombinant CK2 was used (New England Biolabs). The kinase reaction was stopped, and the products were processed as above.

Focus Formation Assay. Hs578T cells were transfected in P100 dishes using Fugene, as described above. After 16 h, cells were plated at 1×10^4/ml in top plugs consisting of complete medium and 0.8% SeaPlaque agarose (FMC Bioproducts, Rockland, ME). Plates were subsequently incubated for 18 days in humidified incubator at 37°C. Cells were stained with 0.5 ml of 0.0005% crystal violet, and colonies were counted using a dissecting microscope.

RESULTS

IKK Complex Kinases Are Constitutively Active in Human Breast Cancer Cell Lines. To determine whether breast cancer cells are characterized by an increase of activity of kinase components of the IKK complex, we first monitored the level of IKKβ activation. Kinase activity levels in untransformed MCF-10F human mammary epithelial cells and breast cancer cell lines Hs578T and carcinogen-transformed D3-1 and BP-1 cells (23) were compared. As controls for the kinase assay, Hs578T cells were treated with TNF-α or transfected with plasmids encoding flag-tagged dominant negative mutated IKKβSS/AA or constitutively active mutant IKKβSS/EE. WCEs were prepared from cultures of the four cell lines at 70% confluence, and samples containing equal amounts of proteins were immunoprecipitated with an IKKβ kinase specific antibody. Alternatively, with the transfected cell extracts, a flag antibody was used. One-third of the immunoprecipitated material was used in *in vitro* phosphorylation assays with full length GST-wtIκB-α as substrate, and protein was labeled with [γ-^{32}P]ATP (Fig. 1*A*, *top panel*). The remainder was subjected to immunoblotting for IKKβ protein or flag epitope, as indicated (Fig. 1*A*, *bottom panel*). Consistent with prior studies (13), ectopic expression of the dominant negative IKKβ SS/AA resulted in reduced IKKβ kinase activity as judged by decreased IκB-α phosphorylation when compared with expression of IKKβSS/EE (Fig. 1*A*). This decrease occurred despite the higher total levels of IKKβ protein (Fig. 1*A*, *bottom panel*), consistent with a dominant negative effect. The IKKβ kinase activity in Hs578T was modestly stimulated by treatment with TNF-α for 10 min (Fig. 1*A*). IKK activity was specific for Ser-32 and Ser-36 of IκB-α, because replacement with alanine at both sites in the GST-IκB-α substrate eliminated phosphorylation (data not shown).

Comparison of the WCEs from the four cell lines indicated IκB-α kinase activity directed by IKKβ was increased in the tumor cell lines compared with MCF-10F (Fig. 1*A*). When normalized for level of expression of IKKβ protein detected in the immunoblot (Fig. 1*A*, *bottom panel*), compared with MCF-10F cells, values obtained in this and a duplicate experiment for the Hs578T, D3-1, and BP-1 tumor cells were 1.6- and 1.4-fold, 2.6- and 2.0-fold, and 1.4- and 1.4-fold, respectively. The increases in all of the cells were statistically significant ($P < 0.05$). A similar analysis was next performed for IKKα. IKKα kinase activity was significantly lower in the untransformed MCF-10F cells compared with all of the three breast cancer cell lines (Fig. 1*B*). Immunoprecipitation of IKKα brought down approximately equivalent amounts of endogenous protein from the four lines (Fig. 1*B*). Compared with the MCF-10F cells, Hs578T, D3-1, and BP-1 tumor cells displayed higher IKKα activities in this and a duplicate experiment of 1.7- and 1.6-fold, 2.0- and 4.2-fold, and 1.9- and 5.0-fold, respectively. Thus, breast tumor cell lines displayed increased IKKα and IKKβ kinase activity compared with untransformed human mammary epithelial cells.

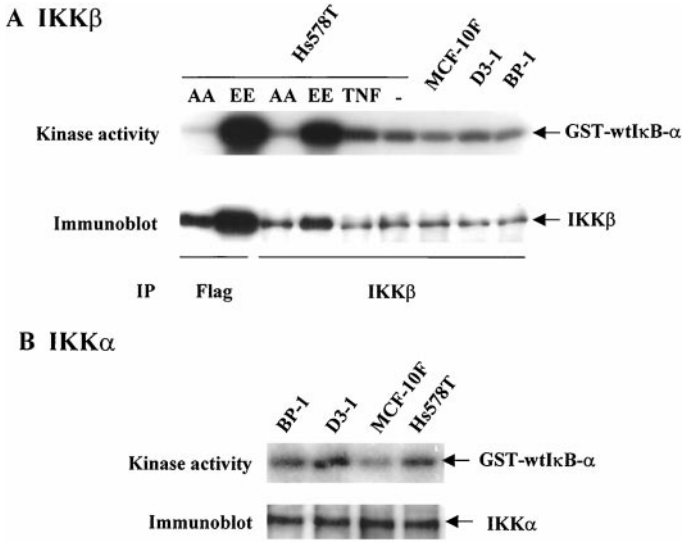

Fig. 1. Analysis of IKK kinase activities in human breast cancer cell lines. *A*, IKKβ analysis. Extracts were prepared from Hs578T, D3-1, and BP-1 breast cancer cells and from untransformed MCF-10F breast epithelial cells. Alternatively, extracts were similarly prepared from Hs578T cells 24 h after transfection with 10-μg vectors pCMV-IKKβSS/AA plasmid (*AA*), expressing dominant negative flag-tagged IKKβSS/AA protein or pCMV-IKKβSS/EE (*EE*), expressing constitutively active IKKβSS/EE protein. Where indicated, extracts were similarly prepared from Hs578T cells treated with 20 ng/ml TNF for 10 min. Equal amounts (150 μg) were immunoprecipitated with an antibody against flag tag or IKKβ, as indicated. Samples (one-third total) were subjected to a kinase assay using GST-wtIκB-α as substrate (*top panel*), whereas the remainders (two-thirds) were subjected to immunoblotting for IKKβ protein. *B*, IKKα analysis. Extracts were prepared as describe above. Equal amounts were immunoprecipitated with an antibody against IKKα, and samples were subjected to the kinase assay using GST-wtIκB-α and immunoblotting for IKKα protein, as above. The data in parts *A* and *B* are representative of one of two experiments.

Kinase Inactive IKKα or IKKβ Inhibits NF-κB Activity in Hs578T Human Breast Cancer Cells. To assess the role of active IKK kinases in the induction of NF-κB seen in the breast tumor cells (5, 7), the effects of inhibition of IKKs on NF-κB activity and binding were evaluated. IKK activity was modulated by transfection with plasmids encoding kinase-inactive mutants IKKαSS/AA and IKKβSS/AA. The transcriptional activity of NF-κB was evaluated by cotransfection with a reporter plasmid driven by wt (E8-CAT) or mutated (negative control, dmE8-CAT) NF-κB-binding elements. In Hs578T cells, functional activation of NF-κB was reduced 1.9-fold ($P < 0.05$) upon transfection with IKKαSS/AA and 2.2-fold ($P < 0.05$) by IKKβSS/AA, compared with the cognate parental vectors (pcDNA3 and pCMV-Neo, respectively; Fig. 2). This observation is consistent with the reduction of IKK kinase activity in transfected cells seen above (Fig. 1*A*). Thus, these findings suggest that IκB-α turnover is mediated by both active IKKα and IKKβ.

Kinase Inactive IKKβ Inhibits NF-κB Activity in D3-1 and BP-1 Human Breast Cancer Cells. We next sought to determine whether inhibition of the IKK complex in the D3-1 and BP-1 cells could similarly reduce NF-κB transcriptional activity and selected the dominant negative IKKβ. D3-1, BP-1, and parental MCF-10F cells were cotransfected with the kinase inactive IKKβSS/AA or parental pCMV-Neo vectors and the wt and dm E8-CAT reporter constructs, as above. In the presence of the parental pCMV-Neo vector, the two transformed lines displayed higher levels of NF-κB activity compared with the MCF-10F cells, as expected (Fig. 3). The increase in activity was greater with the D3-1 than the BP-1 line. NF-κB activity was

greatly reduced in D3-1 and BP-1 cells upon transfection with IKKβSS/AA, whereas no significant change was observed in the MCF-10F cells (Fig. 3). Thus, inhibition of IKKβ activity reduces NF-κB activity in both the D3-1- and BP-1-transformed breast cancer cell lines.

CK2 Kinase Activity Is Increased in Human Breast Cancer Cell Lines. As discussed above, CK2-mediated phosphorylation of IκB-α in the PEST domain has been implicated in the basal turnover of IκB-α in immune cells (14, 18, 19). Thus, to explore the potential role of CK2 on NF-κB levels in breast cancer, a CK2 kinase assay was developed using IκB-α as a specific substrate. GST fusion proteins of either wt IκB-α (GST-wtIκB-α) or mutant Δ269–317 IκB-α (GST-Δ2 IκB-α), which has a deletion of amino acids 269–317 in the COOH-terminal PEST domain (17), were used as positive and negative controls, respectively. Recombinant CK2 enzyme was assayed in the presence of [γ-^{32}P]GTP instead of [γ-^{32}P]ATP, because both ATP and GTP can be used as phosphate donors by CK2 whereas the IKKs can only use ATP. Recombinant CK2 phosphorylated GST-wtIκB-α to a much greater extent than GST-Δ2 IκB-α (Fig. 4*A*). Similar results were obtained with an IκB-α mutant with three point mutations (S283A, T291A, and T299A, GST-3C IκB-α) that had been shown previously (17) to reduce phosphoryl group transfer by CK2 *in vitro* (data not shown).

WCEs were next prepared from Hs578T cells and used directly with GST-IκB-α fusion proteins as substrates in *in vitro* CK2 phosphorylation assays. Kinase assays demonstrated strong preferential phosphorylation of GST-wtIκB-α compared with GST-Δ2 IκB-α or GST-3C IκB-α (Fig. 4*B*). To verify the involvement of CK2, two selective pharmacological inhibitors of CK2, apigenin or emodin, were used either with cells in culture or with cell extracts (29, 30). Apigenin is a plant flavonoid that has been shown to be more effective *in vitro*, although it can also inhibit intracellular CK2 activity (31). Hs578T cells were incubated for 2 h in the presence of 20, 40, or 80 μM apigenin or a volume of carrier DMSO equivalent to 80 μM (Fig. 4*C*, *left panel*). A decrease of 1.5–2-fold in phosphorylation of GST-wtIκB-α was observed with the extracts. Alternatively, WCEs were treated with similar concentrations of apigenin (Fig. 4*C*, *right panel*). A dose-dependent inhibition was noted that was more potent than in cells, as seen previously. The natural plant anthraquinone derivative, emodin, has been shown to inhibit CK2 activity by competitively binding to its ATP-binding site (30). The addition of emodin similarly caused a dose-dependent decrease in CK2 activity both in Hs578T cells or in extracts with almost complete inhibition at 25 μg/ml (Fig. 4*C*). Thus, apigenin and emodin reduced GST-wtIκB-α kinase activity when added to extracts or to cells in culture. Lastly, to confirm the role of CK2 in the observed phosphorylation, a CK2-specific peptide substrate RRREEETEEE was added as competitor (Fig. 4*D*). The

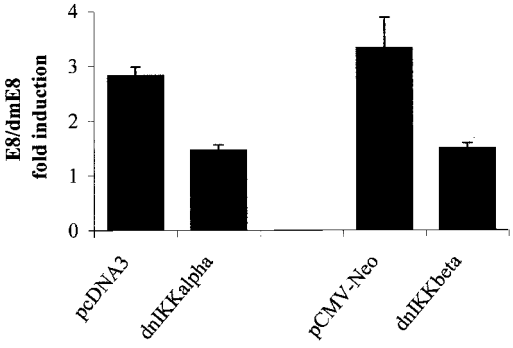

Fig. 2. Inhibition of IKK reduces NF-κB activity in Hs578T cells. Cultures of Hs578T cells were transiently transfected, in duplicate, with 1 μg of E8-CAT (*E8*) or dmE8-CAT (*dmE8*), 0.5 μg of SV40-β-gal in the presence of 1 μg of the indicated vectors. *Left panel*, pcDNA3 parental or pcDNA3-IKKαSS/AA vector, expressing dominant negative IKKα (*dnIKKalpha*) protein. *Right panel*, pCMV-Neo parental or pCMV-IKKβSS/AA vector, expressing dominant negative IKKβ protein (*dnIKKbeta*). After 24 h, cultures were harvested, and samples, normalized for β-gal activity, were assayed for CAT activity. The values for E8-CAT activity are represented as fold induction over dmE8-CAT activity. The data of this and a duplicate experiment are statistically significant ($P < 0.05$).

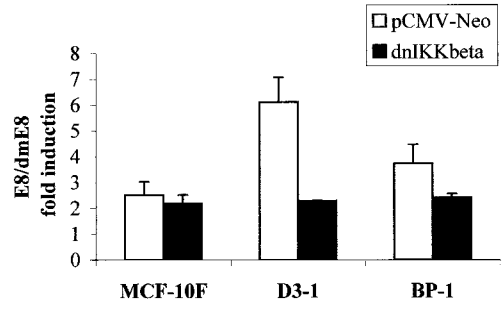

Fig. 3. Inhibition of IKKβ reduces NF-κB activity in D3-1 and BP-1 cells. Cultures of MCF-10F, D3-1, and BP-1 cells were transiently transfected in duplicate with 1 μg of E8 or dmE8, 0.5 μg of SV40-β-gal in the presence of either 1-μg pCMV-Neo parental or pCMV-IKKβSS/AA vector, expressing dominant negative IKKβ protein. After 48 h, cultures were harvested, and samples, normalized for β-gal activity, were assayed for CAT activity. The values for E8-CAT activity are represented as fold induction over dmE8-CAT activity.

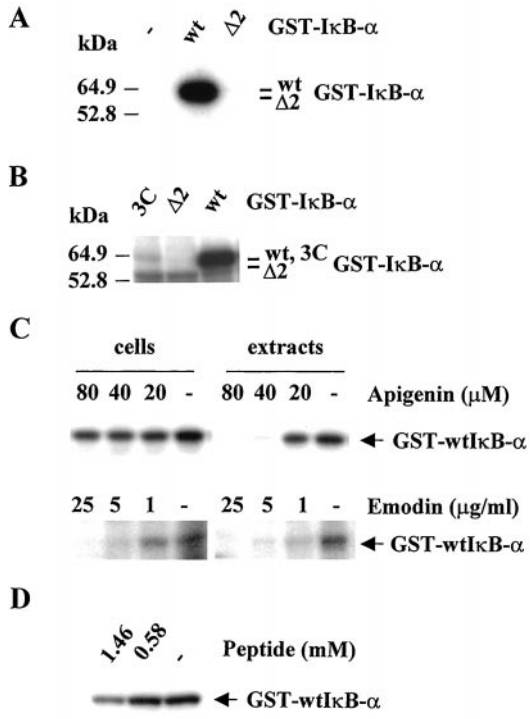

Fig. 4. CK2 kinase assay for IκB-α. *A*, recombinant CK2 assay. Recombinant CK2 (20 units) was incubated with 200 ng of substrate of either GST-wtIκB-α (*wt*) or GST-Δ2 IκB-α (*Δ2*; with deletion of amino acids 269–317 including COOH-terminal PEST domain) in the presence of 10 μCi of [γ-^{32}P]GTP in 25-μl total volume, as described in "Materials and Methods." Proteins were resolved by SDS-PAGE and visualized by autoradiography. Positions of molecular weight protein standards of M_r 64,900 and 52,800 and of the GST-wtIκB-α and GST-Δ2 IκB-α proteins are as indicated. *B*, CK2 activity in Hs578T WCEs. WCEs were prepared from Hs578T cells and used in a CK2 kinase assay, as above, using either GST-wtIκB-α (*wt*), GST-Δ2 IκB-α (*Δ2*), or GST-3C IκB-α (*3C*; with three point mutations at S283A, T291A, and T299A). *C*, inhibition of IκB-α phosphorylation by apigenin or emodin. *Left panel*, Hs578T cells were incubated for 2 h in the presence of 20, 40, or 80 μM apigenin or 1, 5, or 25 μg/ml emodin, dissolved in DMSO or a volume of carrier DMSO equivalent to the highest dose (-). WCEs were assessed for CK2 kinase activity as above. *Right panel*, Hs578T WCEs were used in CK2 kinase assays in the presence of the indicated dose of apigenin or emodin, as above, with GST-wtIκB-α as substrate. *D*, inhibition of IκB-α phosphorylation by the CK2 specific peptide. Hs578T WCEs were used in CK2 kinase assays in the absence (-) or the presence of 0.58 or 1.46 mM CK2-specific peptide substrate RRREEETEEE, added as competitor, and GST-wtIκB-α as substrate.

addition of the peptide substrate RRREEETEEE effectively reduced phosphorylation of the GST-wtIκB-α substrate. Thus, the assay for CK2 activity using GST-IκB-α as a substrate appears to be specific. Furthermore, the results indicate that Hs578T cells display CK2 IκB-α kinase activity.

Next, we compared the relative CK2 activity in the untransformed MCF-10F mammary epithelial parental cells with levels in the carcinogen-transformed lines BP-1 and D3-1 and the Hs578T breast cancer cells. The CK2 kinase activity was clearly higher in all of the tumor cells compared with the MCF-10F line (Fig. 5*A*). Compared with MCF-10F cells, the relative increase in CK2 kinase activity was 2.2-, 2.5-, and 2.2-fold in Hs578T, BP-1, and D3-1 cells, respectively. Increased CK2 activity is most often attributable to increased levels of CK2 protein expression. To assess the relative levels of CK2 protein in the cell lines, immunoblot analysis was performed for the CK2α subunit of CK2 using the WCEs (Fig. 5*B*). The Hs578T, D3-1, and BP-1 cells expressed higher levels of CK2α than MCF-10F cells expressed. Equal loading was confirmed by analysis for β-actin expression. (A slightly lower β-actin level was routinely detected in the Hs578T cells; data not shown.) The results from this and a duplicate experiment were quantified. Compared with the MCF-10F cells, an approximate 2.2±0.3-fold, 1.6±0.1-fold, and 1.6±0.3-fold increase in the level of CK2α protein was found in the Hs578T-, BP-1-, and D3-1-transformed lines, respectively. Thus, IκB-α kinase activity directed by CK2 is increased in these breast tumor cell lines, and this increase can be explained by an increase in levels of CK2α protein.

Inhibition of CK2 Reduces NF-κB Activity in Hs578T Cells. To evaluate the role of CK2 in the constitutive levels of NF-κB in breast cancer cell lines, we chose the CK2-selective inhibitor apigenin. Hs578T cells were incubated for 2.5 h in the presence of 20, 60, or 100 μM apigenin or with the concentration of carrier DMSO equivalent to 100 μM (−apigenin). Nuclear extracts were prepared and analyzed for NF-κB-binding activity by EMSA (Fig. 6*A*). Two major complexes were seen in the untreated Hs578T cells. Successful competition with wt but not mutant oligonucleotide and inhibition upon addition of IκB-α confirmed the specificity of the NF-κB binding (Fig. 6*B*). Antibody supershift analysis indicated that complex 2 represents p50/RelA, and complex 1 is a homodimer of p50, consistent with our previous observations with this line (7). Apigenin reduced the formation of both bands 1 and 2 in a dose-dependent fashion. Little change was noted at 20 μM, whereas a substantial decline in the upper p50/RelA complex was observed at 60 μM. At a concentration of 100 μM, reduced formation of both complexes was noted. In contrast, little effect was seen on the binding of Sp1 (Fig. 6*A*). When these results were scanned and normalized to the

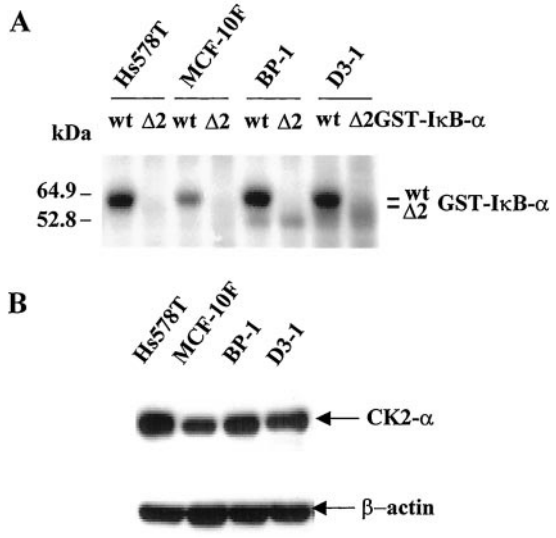

Fig. 5. CK2 activity is elevated in breast cancer cell lines. WCEs were prepared from cultures of Hs578T, D3-1, and BP-1 breast cancer cells and untransformed MCF-10F cells at 70% confluence. *A*, CK2 activity. Samples (10 μg) were subjected to a CK2 kinase assay, as above, using either GST-wtIκB-α (*wt*) or GST-Δ2 IκB-α (*Δ2*) as substrate. *B*, CK2α protein levels. Samples (50 μg) were separated by SDS-PAGE, and the same blot was subjected to immunoblot analysis for CK2α and β-actin levels.

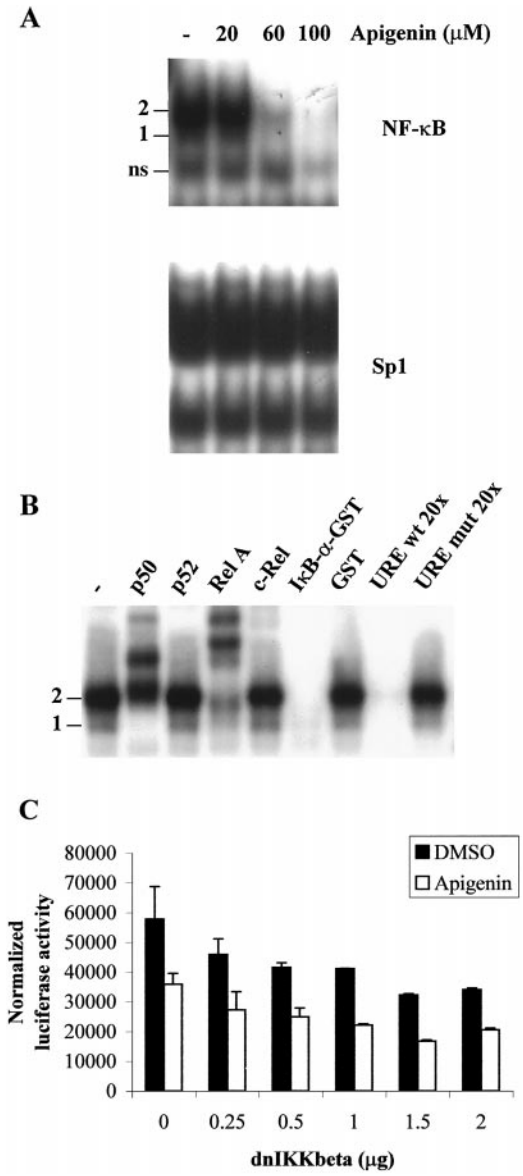

Fig. 6. Apigenin inhibits CK2 activity and NF-κB binding and transcription in Hs578T cells. *A*, binding assay. Cells were incubated for 150 min in the presence of 20, 60, or 100 μM apigenin dissolved in DMSO or a volume of carrier DMSO equivalent to 100 μM (-). Nuclear extracts were prepared, and samples (5 μg) were subjected to EMSA using oligonucleotides containing either NF-κB- or Sp1-binding elements. *B*, supershift analysis. Samples of Hs578T cell nuclear extracts (2.5 μg) were incubated overnight at 4°C in the absence (-) or the presence of 2 μg of antibody against the p65 subunit, p50 subunit, p52 subunit, c-Rel NF-κB subunit, 250 ng of IκB-α-GST, or GST protein and subjected to EMSA for NF-κB as described in "Materials and Methods." To test for specificity of binding, the binding reaction was incubated with 20× excess unlabeled wt (*URE wt*) or mutant URE oligonucleotide (*URE mut*), as indicated. NF-κB bands 1 and 2 contain p50 homodimer and p50/RelA heterodimer complexes, respectively. *C*, transcriptional activity. Cultures were transiently transfected in duplicate with 1 μg of NF-κB element-driven luciferase reporter construct and 0.5 μg of SV40-β-gal in the presence of the indicated amount of pCMV-IKKβSS/AA vector, expressing dominant negative IKKβ protein and enough pCMV-Neo parental DNA to maintain a constant amount of 2 μg. After 16 h, cells were incubated for 6 h in the presence of 60 μM apigenin dissolved in DMSO (□) or equivalent volume of carrier DMSO (■). Cultures were then harvested, normalized for β-gal activity, and assayed for luciferase activity. The values for luciferase normalized for β-gal activity are represented.

Sp1-binding activity, a 70% decrease in classical NF-κB was noted with 60 μM apigenin. At 100 μM apigenin, decreases of 95% and 80% in p50/RelA and p50 homodimers, respectively, were observed. Next, we verified that the NF-κB transcriptional activity is decreased by inhibition of CK2 using either apigenin or emodin. After transfection with vectors expressing an NF-κB element luciferase reporter plus SV40-β-gal, for normalization, cells were incubated overnight and then treated with either 60 μM apigenin or 25 μg/ml emodin. The treatments were performed for 6 h to ensure sufficient time for the transcriptional activity to decline and for decay of the luciferase protein. Apigenin treatment resulted in an approximate 43% decline in NF-κB activity (39.2 ± 3.8% and 45.6 ± 6.1% in two separate experiments). Emodin treatment caused an approximate 30% drop in NF-κB activity (35.9 ± 3.6 and 24.9 ± 1.8% in two separate experiments). Taken together, these results suggest that inhibition of CK2 leads to a reduction of nuclear NF-κB-binding activity in Hs578T breast cancer cells.

To assess whether inhibition of IKK and CK2 activities would be additive, NF-κB luciferase reporter activity was measured in Hs578T cells cotransfected with increasing doses of the dominant negative IKKβ expression vector and treated for 6 h with 60 μM apigenin. A dose-dependent drop in NF-κB activity was seen with the dominant negative IKKβ expression vector, which reached a plateau at 40% inhibition with a 1.5-μg expression vector DNA (Fig. 6*C*). Furthermore, addition of apigenin increased the extent of inhibition (by 37 to 48%) at every dose of dominant negative IKKβ expression vector used; *e.g.*, NF-κB activity was inhibited a total of 71% in cells treated with apigenin and 1.5-μg dominant negative IKKβ expression vector DNA. These results indicate that the effects of IKK and CK2 on NF-κB are additive.

Inhibition of NF-κB Reduces Anchorage-independent Growth of Hs578T Human Breast Cancer Cells. Next, we assessed the role of NF-κB in transformed phenotype using anchorage-independent growth of Hs578T breast cancer cells. Because the dominant negative ΔC IKKAP1 construct, encoding a COOH-terminal deletion-mutant of IKKγ/NEMO/IKKAP1, has been shown to inhibit activities of both IKKα and IKKβ (8–10), it was selected for analysis here. Using transfection into Hs578T cells, we first confirmed the ability of expression of the ΔC IKKAP1 vector to decrease classical NF-κB-binding levels by 70% (data not shown). Cultures of Hs578T cells were then transfected in triplicate with parental pcDNA3EE vector or with 3- or 10-μg ΔC IKKAP1 vector and assessed for growth in soft agar. The colonies/high power field were as follows: for pcDNA3EE, 132 ± 34; 3-μg ΔC IKKAP1, 84 ± 16; and 10-μg ΔC IKKAP1, 57 ± 11. As expected, transfection with a vector expressing full-length IKKγ/NEMO/IKKAP1 had no effect on NF-κB activity or colony formation (data not shown). Thus, a dose-dependent reduction in colony numbers was seen with transfection of the ΔC IKKAP1 vector compared with the parental vector. Taken together, these results indicate that breast cancer cells display a substantial increase in activity of kinases that induce NF-κB activity and that this induction can promote the transformed phenotype, as measured by anchorage-independent growth of these cells.

Primary Breast Cancers, Displaying Increased NF-κB-Binding Activity, Have Either Increased IKK or CK2 Kinase Activities. Next, we asked whether these three IκB kinases are activated in primary human breast cancer specimens and whether NF-κB induction correlates with kinase activation. Two sets of human primary breast tumors were studied. The pathological characteristics and steroid receptor data that were available from these patient cases (Table 1; and data not shown) show that 9 of 16 were estrogen receptor (ER) positive, 2 of 16 were ER intermediate, and 5 of 16 were ER negative, whereas 14 of 16 were progesterone receptor (PR) positive. Nuclear extracts were prepared from frozen breast tumors and used for NF-κB binding by EMSA. Because potential contamination with hematopoietic cells could significantly affect the analysis, our strategy was to also test for such contamination using a binding assay for PU.1 and TCF-1, which are present in B lymphocytes, neutrophils, mast or myeloid cells, and T cells. Fig. 7 and Fig. 8 show data obtained from the analysis of the first group of six patient samples, and Table 1 presents the findings for every PU.1- and TCF-1-negative sample from the two sets analyzed.

In analysis of the first six specimens, binding of full-length PU.1 protein was detected in sample 6679, and a clipped form of PU.1 protein was detected in patients 6680, 6921, and 6613 (Fig. 7*A*, *left panel*). These findings were confirmed using immunoblot analysis (data not shown). Compared with Jurkat T cells, only low and comparable levels of binding to TCF-1 was detected in the tumor extracts (data not shown). Two of the samples (6731 and 6712) that tested negative for PU.1 were found to express relatively high levels of NF-κB binding (Fig. 7*A*, *right panel*). The gels were subjected to densitometry, and the results of the analysis of all of the samples that tested negative for PU.1 and TCF-1 are presented in Table 1.

To identify which NF-κB subunits are present in the nuclear extracts, immunoblot analysis was performed using antibodies specific for RelA (p65), c-Rel, p50, and p52 (Fig. 7*B*). Both samples 6712 and 6731 displayed nuclear p65 proteins, as did 6679. When c-Rel was assessed, only 6731 displayed detectable levels of expression. The p50 or p52 subunits were detected in both patient samples. (The p50 in sample 6712 had a slightly faster mobility than p50 in sample 6731.) Sample 6731 displayed somewhat more expression of p50 (band 1) than p52, whereas sample 6712 expressed more p52 than p50 (Fig. 7*B*). Thus, sample 6731 contains transactivating subunits RelA and c-Rel, whereas 6712 contains RelA. The data for expression of RelA and c-Rel in the two sets of patient samples are summarized in

Table 1 *IKK and CK2 kinase activity in primary human breast specimens*[a]

Patient	Tumor type	ER/PR levels (fmol/mg)	NF-κB binding[b]	RelA[b]	c-Rel[b]	Kinase activity[b]		
						IKKα	IKKβ	CK2
6712[c]	Right infiltrating ductal cancer	006/000	3939	2578	-[d]	129	178	2446
6731[c]	Right breast mass	026/162	4868	1137	687	432	114	1809
6885	Not available	002/010	114	-	-	230	316	6143
8357	Right invasive lobular cancer	000/000	970	-	-	-	nd	341
8359	Ductal cancer	001/004	646	-	-	182	276	415
8360	Left infiltrating ductal cancer	051/035	344	-	-	-	294	231
8361	Right breast mass	022/006	6022	4764	74	133	1164	1106
8364	Not available	123/372	1866	1713	-	355	1395	819
8385	Metastatic cancer	018/011	2840	2938	652	247	980	758
6698	Left breast mass	367/033	3689	-	-	137	243	3902

[a] Table presents results observed with two groups of 6 and 10 primary human breast specimens. Only data from PU.1- and TCF-1-negative tumors are shown. Data were normalized by using the same cell extracts from WEHI 231 B cells in every assay (data not shown).
[b] Densitometry, arbitrary units.
[c] From the first series of patients.
[d] -, no expression/activity detected; nd, not done.

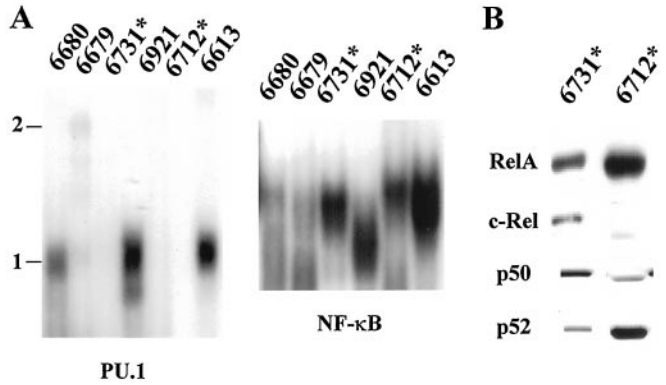

Fig. 7. NF-κB activation in patient breast cancer specimens. *A*, nuclear extracts were prepared from the indicated anonymous patient specimens, and samples (5 μg) were used in EMSA with oligonucleotides specific for PU.1, which is present in B lymphocytes, neutrophils, and mast and myeloid cells, and for NF-κB. The positions of the intact and partially degraded PU.1 proteins are indicated as bands 2 and 1, respectively; *, two specimens (6731 and 6712) that appeared negative for PU.1 and positive for NF-κB binding. *B*, samples of the nuclear extracts from *A* (20 μg) for patient samples 6631 and 6712 were subjected to immunoblot analysis for expression of RelA, c-Rel, p50, and p52. *Lanes* for the two samples were taken from the same gels.

Table 1. Essentially, all of the tumors tested positive for the p50 or p52 subunit (data not shown). Of the 10 PU.1/TCF-1-negative breast cancer samples characterized, 3 displayed only low levels of NF-κB binding, whereas 1 had a minimally elevated level, and 6 showed substantially elevated levels of NF-κB binding.

Cytoplasmic extracts from tumors of these patients were then tested for CK2 IκB-α kinase activity using GST-wtIκB-α as substrate (Fig. 8*A*; and data not shown). Two of the six primary tumor samples from the first set of patients showed elevated CK2 IκB-α kinase activity, patients 6731 and 6712 (Fig. 8*A*). As a negative control for the kinase assay, two samples were tested with GST-Δ2 IκB-α as substrate. The extracts failed to phosphorylate this IκB-α protein containing a deletion of the PEST domain sequences. Lastly, to confirm the specificity of the assay for CK2, the selective inhibitor apigenin was added to the reaction with wtIκB-α as substrate. Apigenin dramatically reduced IκB-α phosphorylation with samples 6680 and 6731, confirming the reactions were mediated by CK2. The results from the two sets of patients were quantified by densitometry; the data for the PU.1/TCF-1-negative samples are presented in Table 1. Three of the specimens displayed low levels of CK2 activity (between 231 and 415 densitometry units), whereas the remaining specimens had either modestly increased (758 to 1106 densitometry units) or substantially elevated levels (1809 to 6143 densitometry units; Table 1). The three specimens with low CK2 also displayed low or minimally elevated NF-κB binding. Six samples displayed elevated levels of CK2 and NF-κB binding, whereas only one specimen (6885) showed high IκB-α CK2 kinase activity without detectable high NF-κB nuclear activity.

Activities of the IKKα and IKKβ kinases were evaluated after immunoprecipitation with their specific antibodies, and the data are presented in Fig. 8, *B* and *C*, respectively. The results for PU.1/TCF-1-negative tumors are shown in Table 1. Tumor samples 6731 and 8364 exhibited modestly increased levels of IκB-α kinase activity directed by IKKα and increased NF-κB-binding activity. Consistent with data obtained with tumor cell lines, increased IKKα activity in primary tumors did not seem to be attributable to increased levels of protein expression (Fig. 8*B*; and data not shown). In the analysis of IKKβ kinase activity, most of the samples yielded values between approximately 100 and 300 densitometry units; however, cytosolic extracts from three specimens (8361, 8364, and 8385) displayed greatly elevated IKKβ activity (1164, 1395, and 980 densitometry units, respectively). All of these three specimens displayed high NF-κB-binding activity. No phosphorylation was detected using a mutant of IκB-α at Ser-32 and Ser-36 as substrate, confirming the specificity of the kinase assay (data not shown). Thus breast cancers display activation of CK2, IKKα, IKKβ, or various combinations of these kinases, which correlate and are, therefore, likely responsible for the aberrant NF-κB activation in these primary tumors.

DISCUSSION

In this study, we show that human breast cancer cell lines and primary human breast tumor specimens display elevated CK2, IKKβ, and/or IKKα IκB kinase activities. Inhibition of any of these activities in the breast cancer cells resulted in reduced functional NF-κB/Rel. Reduced NF-κB levels, via expression of a dominant negative IKKγ/NEMO/IKKAP1 to repress the IκB kinase complex, resulted in loss of soft agar colony formation ability. Previously, we and others (5, 6) demonstrated that primary breast cancer samples from patients or from a carcinogen-induced rodent model, as well as cell lines in culture, are typified by aberrant activation of NF-κB. In contrast, only low levels of NF-κB/Rel factors are present in the nuclei of normal breast epithelial cells (5, 7). These results, which were extended to additional patients here, have been confirmed recently by other laboratories (6, 32–34). For example, Cogswell *et al.* (34) showed that breast tumors compared with adjacent normal tissues display increased mRNA and protein expression of the NF-κB subunits p50,

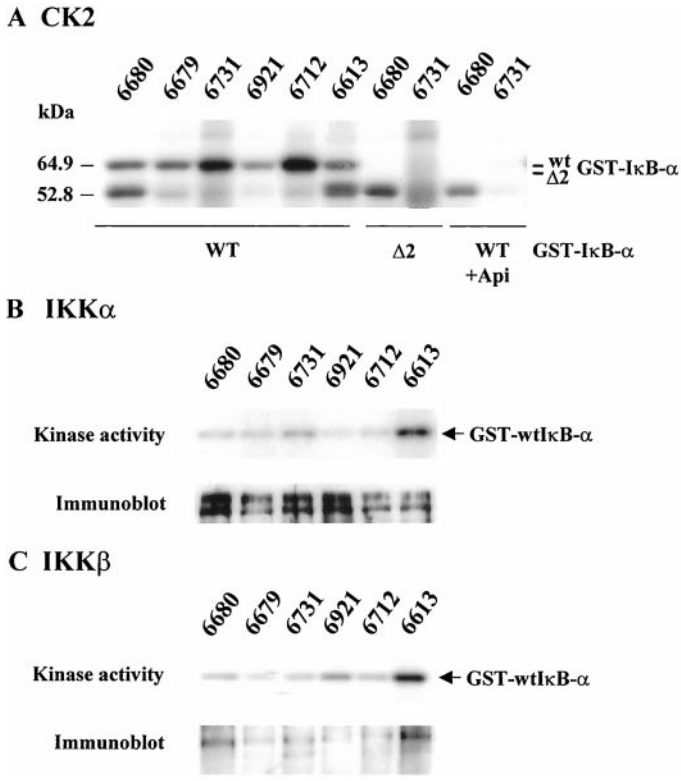

Fig. 8. CK2 and IKK activity in patient breast cancer specimens. *A*, CK2 kinase assay was performed with samples (20 μg) of cytoplasmic extracts from the indicated coded patient samples and with GST-wtIκB-α (*WT*) as substrate or as a negative control GST-Δ2 IκB-α (*Δ2*; with deletion of amino acids 269–317 including COOH-terminal PEST domain). As an additional control for CK2 specificity of the assay, inhibition of the phosphorylation of GST-wtIκB-α with the selective CK2 inhibitor apigenin was carried. *B*, equal amounts (150 μg) of cytoplasmic extracts were immunoprecipitated with an antibody against IKKα. Portions (one-third total) were subjected to the kinase assay using GST-wtIκB-α (*top panel*), whereas the remainder (two-thirds) was subjected to immunoblotting for IKKα protein. *C*, equal amounts (150 μg) of cytoplasmic extracts were immunoprecipitated with an antibody against IKKβ. Samples (one-third total) were subjected to kinase assay using GST-wtIκB-α, and the remainder (two-thirds) was subjected to immunoblotting for IKKβ protein.

p52, c-Rel, as well as Bcl-3, which could enhance p50 activity. As discussed below, the mechanisms mediating activation of NF-κB/Rel factors, which regulate expression of genes that control cell proliferation, survival, and transformation, are under investigation in many laboratories. In this study for the first time, aberrant induction of IKKα, IKKβ, and CK2, key kinases that regulate IκB stability and NF-κB/Rel activation, is demonstrated in breast cancer.

Aberrant nuclear NF-κB activity has been reported in multiple cancers, including the human cutaneous T-cell lymphoma HuT-78, primary adult T-cell leukemias, acute lymphoblastic leukemia, and pancreatic adenocarcinomas (2, 35–38). In hematopoietic and solid tumors, mutations or modulation in the expression of the IκB-α protein, as well as amplification, overexpression, or gene rearrangement of the *nfkb1*, *nfkb2*, *bcl3*, *c-rel*, or *relA* genes have been noted (2). Mutations of IκB-α have been reported that decrease affinity for NF-κB in Hodgkin lymphomas (39), whereas other studies (7, 40, 41) have correlated increased NF-κB expression in tumor cells of various types with decreased stability of IκB proteins. Furthermore, products of several oncogenes have been found to activate NF-κB. In breast cancer, overexpression of the HER-2/Neu receptor, which is found in approximately 30% of patients, or of the epidermal growth factor receptor was found to induce p50/RelA complexes specifically (42–44). Consistent with these findings, in our two studies, nuclear RelA was observed in several primary human breast tumor samples. In contrast, only low levels of RelA were seen by Cogswell *et al.* (34), which likely reflect differences in the patient populations studied. Oncogenic Raf and Ras proteins (27, 45, 46) and the HTLV-1 tax protein (4, 47) have also been found to induce NF-κB activity in multiple cell types. The details of pathways responsible for the increased NF-κB in tumor cells are only beginning to be resolved.

IKK kinase has been implicated in the persistent NF-κB nuclear activity in HTLV1-infected T lymphocytes (48), Hodgkin lymphomas (40), and melanoma cells (41). Recently, we demonstrated that activation of NF-κB by oncogenic Raf is mediated by a Mek to IKKβ pathway (49). Ras has been found to induce NF-κB via phosphatidylinositol 3′-kinase to IKKα and also via the Raf pathway (49). In this study, all of the breast cancer cell lines studied displayed increased activity of both IKKα and IKKβ. Furthermore, tumors from three patients were shown to have very high levels of IKKβ activity, and tumors from two patients displayed modestly elevated levels of IKKα. These latter findings suggest that differential pathways leading to increased activation of these kinases occurred in the various tumor specimens. It should be noted, however, that both IKKα and IKKβ can form heterodimers or homodimers that can phosphorylate IκB-α (13). Unfortunately, the amino acids comprising the activation loop serines are nearly identical for IKKα and IKKβ, making it difficult to develop antibodies specific for the active forms of IKKα or IKKβ kinases. In future studies, the use of two-dimensional gel electrophoresis will be explored to determine whether the increase in activity results from activation of IKKα or IKKβ or from both kinases.

Elevated CK2 levels have been reported in multiple human tumors, including squamous cell carcinoma, colorectal tumors, and leukemias (50–52). In one study, immunohistochemistry suggested that human breast tumors also had elevated levels of CK2 protein (53). In this study, we find that high levels of CK2 activity were found in many of the breast tumor specimens and in all of the cancer cell lines studied. Enforced CK2 expression in T cells within mice was sufficient to induce T lymphomas (54). More recently, we have found that transgenic overexpression of CK2α in the mammary gland leads to breast tumors in mice (55). Consistent with the work presented in this study, cells derived from these mammary tumors were found to contain functional NF-κB (55). Interestingly, the CK2α promoter has been found to be regulated by NF-κB (56). Thus, a positive feedback loop regulation may play a role in the elevated CK2 and NF-κB expression seen in breast cancer. To date, there are no reliable dominant negative kinase inactive versions of CK2, which may reflect the complex nature of the tetramer interaction. Thus, to inhibit CK2 we used the selective inhibitors, apigenin and emodin (29–31). Apigenin or emodin treatment reduced constitutive NF-κB activity, suggesting CK2-mediated phosphorylation of IκB-α is important in the aberrant NF-κB activation seen in breast cancer cells. These findings are consistent with previous studies (57, 58) showing the inhibition of NF-κB activity induced by lipopolysaccharide or interferon-γ in macrophages or by TNF-α in endothelial cells can be repressed by apigenin or emodin, although no analysis of the CK2 activity was done in these reports. The mechanism by which CK2 modulates IκB-α stability remains to be determined, and the involvement of proteasome-dependent and -independent pathways have been reported (14, 17, 59). Overall, more work is required to determine the extent of involvement of these kinases in various cancers and the mechanisms of their activation.

Transfection of Hs578T cells with a dominant negative IKKγ/NEMO/IKKAP1 reduced NF-κB binding, transcriptional activity, and colony formation in soft agar. Transfection of the dominant negative IKKβ vector also inhibited colony formation, although more modestly, consistent with the fact that it only partially affects IKK activity (data not shown). Using other methods to inhibit NF-κB/Rel factors, either a decrease in proliferation or survival has been seen. For example, Higgins *et al.* (60) demonstrated inhibition of growth of diverse tumor cells *in vivo* using antisense oligonucleotides to the p65 subunit. We demonstrated that microinjection of IκB-α in Hs578T breast cancer or WEHI 231 B cells in culture led to apoptosis of 20–30% of cells (5, 61). In preliminary experiments, inducible expression of the IKKβ dominant negative protein in stable D3-1 and Hs578T cell lines was found to reduce NF-κB activity and growth rate (data not shown). NF-κB/Rel factors are known to control genes that mediate cell proliferation (*e.g.*, c-*myc* and cyclin D1; Refs. 4, 62), survival (*e.g.*, IAP, Bcl-X_L, and Bcl-2; Refs. 61–63), and metastasis (*e.g.*, urokinase plasminogen activator and metalloproteinases; Refs. 63, 64). Thus, the different responses seen between the various methods of inhibition and cell types likely result from the magnitude, extent, and kinetics of inhibition of NF-κB, as well as upon the nature of the subunits induced and coacting factors present that control expression of genes regulated by this family.

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REFERENCES

1. Siebenlist, U., Franzoso, G., and Brown, K. Structure, regulation, and function of NF-κB. Annu. Rev. Cell Biol., *10:* 405–455, 1994.
2. Rayet, B., and Gelinas, C. Aberrant *Rel/NF-κB* genes and activity in human cancer. Oncogene, *18:* 6938–6947, 1999.
3. Smith, M. R., and Greene, W. C. Identification of HTLV-1 tax trans-activator mutants exhibiting novel transcriptional phenotypes. Genes Dev., *4:* 1875–1885, 1990.
4. Duyao, M. P., Buckler, A. J., and Sonenshein, G. E. Interaction of an NF-κB-like factor with a site upstream of the c-*myc* promoter. Proc. Natl. Acad. Sci. USA, *87:* 4727–4731, 1990.
5. Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Investig., *100:* 2952–2960, 1997.
6. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol., *17:* 3629–3639, 1997.
7. Kim, D. W., Sovak, M. A., Zanieski, G. J., Nonet, G., Romieu-Mourez, R., Lau, A. W., Hafer, L. J., Yaswen, P., Stampfer, M., Rogers, A. E., Russo, J., and

Sonenshein, G. E. Activation of NF-κB/Rel occurs early during neoplastic transformation of mammary cells. Carcinogenesis (Lond.), *21:* 871–879, 2000.
8. Mercurio, F., and Manning, A. M. Multiple signals converging on NF-κB. Curr. Opin. Cell Biol., *11:* 226–232, 1999.
9. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. IKK-γ is an essential regulatory subunit of the IκB kinase complex. Nature (Lond.), *395:* 297–300, 1998.
10. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Ray, R. J., and Israel, A. Complementation cloning of NEMO, a component of IκB kinase complex essential for NF-κB activation. Cell, *93:* 1231–1240, 1988.
11. Mercurio, F., Murray, B. W., Shevchenko, A., Bennett, B. L., Young, D. B., Li, J. W., Pascual, G., Motiwala, A., Zhu, H., Mann, M., and Manning, A. M. IκB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. Mol. Cell. Biol., *19:* 1526–1538, 1999.
12. DiDonato, J., Hayakawa, A., Rothwarf, D. M., Zandi, E., and Karin, M. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature (Lond.), *388:* 548–554, 1997.
13. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J. W., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science (Wash DC), *278:* 860–865, 1997.
14. Pando, M. P., and Verma, I. M. Signal dependent and independent degradation of free and NF-κB bound IκB-α. J. Biol. Chem., *14:* 21278–21286, 2000.
15. Baeuerle, P. A., and Henkel, T. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol., *12:* 141–179, 1994.
16. Barroga, C. F., Stevenson, J. K., Schwarz, E. M., and Verma, I. M. Constitutive phosphorylation of IκB-α by casein kinase II. Proc. Natl. Acad. Sci. USA, *92:* 7637–7641, 1995.
17. Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. Phosphorylation of IκB-α in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. Mol. Cell. Biol., *16:* 1401–1409, 1996.
18. McElhinny, J. A., Trushin, S. A., Bren, G. D., Chester, N., and Paya, C. V. Casein kinase II phosphorylates IκB-α at S-283, S-289, S-293, and T-291 and is required for its degradation. Mol. Cell. Biol., *16:* 899–906, 1996.
19. Schwarz, E. M., Van Antwerp, D., and Verma, I. M. Constitutive phosphorylation of IκB-α by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IκB-α. Mol. Cell. Biol., *16:* 3554–3559, 1996.
20. Allende, J. E., and Allende, C. C. Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. FASEB J., *9:* 313–323, 1995.
21. Guerra, B., and Issinger, O. G. Protein kinase CK2 and its role in cellular proliferation, development, and pathology. Electrophoresis, *20:* 391–408, 1999.
22. Miyamoto, S., Seufzer, B. J., and Shumway, S. D. Novel IκB-α proteolytic pathway in WEHI 231 immature B cells. Mol. Cell. Biol., *18:* 19–29, 1998.
23. Calaf, G., and Russo, J. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis (Lond.), *14:* 483–492, 1993.
24. Kimmelman, A., Tolkacheva, T., Lorenzi, M. V., Osada, M., and Chan, A. M-L. Identification and characterization of R-*ras 3:* a novel member of the *RAS* gene family with a nonubiquitous pattern of tissue distribution. Oncogene, *15:* 2675–2685, 1997.
25. Dolwick, K. M., Swanson, H. I., and Bradfield, C. A. *In vitro* analysis of Ah receptor domains involved in ligand-activated DNA recognition. Proc. Natl. Acad. Sci. USA, *90:* 8566–8570, 1993.
26. Sambucetti, L. C., Cherrington, J. M., Wilkinson, G. W., and Mocarski, E. S. NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T-cell stimulation. EMBO J., *8:* 4251–4258, 1989.
27. Arsura, M., FitzGerald, M. J., Fausto, N., and Sonenshein, G. E. Nuclear Factor-κB/Rel blocks transforming growth factor β1-induced apoptosis of murine hepatocyte cell lines. Cell Growth Differ., *8:* 1049–1059, 1997.
28. van de Wetering, M., Oosterwegel, M., Dooijes, D., and Clevers, H. Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. EMBO J., *10:* 123–132, 1991.
29. Critchfield, J. W., Coligan, J. E., Folks, T. M., and Butera, S. T. Casein kinase II is a selective target of HIV-1 transcriptional inhibitors. Proc. Natl. Acad. Sci. USA, *94:* 6110–6114, 1997.
30. Battistutta, R., Sarno, S., De Molliner, E., Papinutto, E., Zanotti, G., and Pinna, L. A. The replacement of ATP by the competitive inhibitor emodin induces conformational modifications in the catalytic site of the protein kinase CK2. J. Biol. Chem., *275:* 29618–29622, 2000.
31. Ford, H. L., Landesman-Bollag, E., Dacwag, C. S., Stukenberg, P. T., Pardee, A. B., and Seldin, D. C. Cell cycle-regulated phosphorylation of the human SIX1 homeodomain protein. J. Biol. Chem., *275:* 22245–22254, 2000.
32. Dejardin, E., Deregowski, V., Chapelier, M., Jacobs, N., Gielen, J., Merville, M. P., and Bours, V. Regulation of NF-κB activity by IκB-related proteins in adenocarcinoma cells. Oncogene, *18:* 2567–2577, 1999.
33. Ricca, A., Biroccio, A., Del Bufalo, D., Mackay, A. R., Santoni, A., and Cippitelli, M. bcl-2 overexpression enhances NF-κB activity and induces mmp-9 transcription in human MCF7(ADR) breast-cancer cells. Int. J. Cancer, *86:* 188–196, 2000.
34. Cogswell, P., Guttridge, D. C., Funkhouser, W. K., and Baldwin, A. S., Jr. Selective activation of NF-κB subunits in human breast cancer. Oncogene, *19:* 1123–1131, 2000.
35. Giri, D. K., and Aggarwal, B. B. Constitutive activation of NF-κB causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. J. Biol. Chem., *273:* 14008–14014, 1998.
36. Mori, N., Fujii, M., Ikeda, S., Yamada, Y., Tomonaga, M., Ballard, D. W., and Yamamoto, N. Constitutive activation of NF-κB in primary adult T-cell leukemia cells. Blood, *93:* 2360–2368, 1999.
37. Wang, W., Abbruzzese, J. L., Evans, D. B., Larry, L., Cleary, K. R., and Chiao, P. J. The NF-κB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin. Cancer Res., *5:* 119–127, 1999.
38. Kordes, U., Krappman, D., Heissmeyer, V., Ludwig, W. D., and Scheidereit, C. Transcription factor NF-κB is constitutively activated in acute lymphoblastic leukemia cells. Leukemia (Baltimore), *14:* 399–402, 2000.
39. Emmerich, F., Meiser, M., Hummel, M., Demel, G., Foss, H. D., Jundt, F., Mathas, S., Krappmann, D., Scheidereit, C., Stein, H., and Dorken, B. Overexpression of IκB α without inhibition of NF-κB activity and mutations in the *IκB-α* gene in Reed-Sternberg cells. Blood, *94:* 3129–3134, 1999.
40. Krappmann, D., Emmerich, F., Kordes, U., Scharschmidt, E., Dorken, B., and Scheidereit, C. Molecular mechanisms of constitutive NF-κB/Rel activation in Hodgkin/Reed-Sternberg cells. Oncogene, *18:* 943–953, 1999.
41. Devalaraja, M. N., Wang, D. Z., Ballard, D. W., and Richmond, A. Elevated constitutive IκB kinase activity and IκB-α phosphorylation in Hs294T melanoma cells lead to increased basal MGSA/GRO-α transcription. Cancer Res., *59:* 1372–1377, 1999.
42. Biswas, D. K., Cruz, A. P., Gansberger, E., and Pardee, A. B. Epidermal growth factor-induced nuclear factor-κB activation. Proc. Natl. Acad. Sci. USA, *97:* 8542–8547, 2000.
43. Zhou, B. P., Hu, M. C., Miller, S. A., Yu, Z., Zia, W., Lin, S. Y., and Hung, M. C. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-κB pathway. J. Biol. Chem., *275:* 8027–8031, 2000.
44. Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R. J., and Sonenshein, G. E. Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IκB-α that can be inhibited by the tumor suppressor PTEN. Oncogene, *20:* 1287–1299, 2001.
45. Finco, T. S., Westwick, J. K., Norris, J. L., Beg, A. A., Der, C. J., and Baldwin, A. S., Jr. Oncogenic Ha-Ras-induced signaling activates NF-κB transcriptional activity, which is required for cellular transformation. J. Biol. Chem., *272:* 24113–24116, 1997.
46. Jo, H., Zhang, H., McKinsey, T. A., Shao, J., Beauchamp, R. D., Ballard, D. W., and Liang, P. NF-κB is required for H-ras oncogene induced abnormal cell proliferation and tumorigenesis. Oncogene, *19:* 841–849, 2000.
47. Geleziunas, R., Ferrell, S., Lin, X., Mu, Y., Cunningham, E. T., Jr., Grant, M., Connelly, M. A., Hambor, J. E., Marcu, K. B., and Greene, W. C. Human T-cell leukemia virus type 1 Tax induction of NF-κB involves activation of IκB kinase α (IKKα) and IKKβ cellular kinases. Mol. Cell. Biol., *18:* 5257–5262, 1998.
48. Chu, Z-L., DiDonato, J. A., Hawiger, J., and Ballard, D. W. The tax oncoprotein of human T-cell leukemia virus type 1 associates with and persistently activates IκB kinases containing IKKα and IKKβ. J. Biol. Chem., *273:* 15891–15894, 1998.
49. Arsura, M., Hofmann, C. S., Golay, J., Introna, M., and Sonenshein, G. E. A-*myb* rescues murine B-cell lymphomas from IgM-receptor-mediated apoptosis through c-*myc* transcriptional regulation. Blood, *96:* 1013–1020, 2000.
50. Gapany, M., Faust, R. A., Tawfic, S., Davis, A., Adams, G. L., and Ahmed, K. Association of elevated protein kinase CK2 activity with aggressive behavior of squamous cell carcinoma of the head and neck. Mol. Med., *1:* 659–666, 1995.
51. Phan-Dinh-Tuy, F., Henry, J., Boucheix, C., Perrot, J. Y., Rosenfeld, C., and Kahn, A. Protein kinases in human leukemic cells. Am. J. Hematol., *19:* 209–218, 1985.
52. Sietz, G., Munstermann, U., Schneider, H. R., and Issinger, O. G. Characterization of casein kinase II in human colonic carcinomas after heterotransplantation into nude mice. Biochem. Biophys. Res. Commun., *163:* 635–641, 1989.
53. Munstermann, U., Fritz, G., Seitz, G., Lu, Y. P., Schneider, H. R., and Issinger, O. G. Casein kinase II is elevated in solid human tumours and rapidly proliferating non-neoplastic tissue. Eur. J. Biochem., *189:* 251–257, 1990.
54. Seldin, D. C., and Leder, P. Casein kinase II α transgene-induced murine lymphoma: relation to theileriosis in cattle. Science (Wash DC), *267:* 894–897, 1995.
55. Landesman-Bollag, E., Romieu-Mourez, R., Song, D. H., Sonenshein, G. E., Cardiff, R. D., and Seldin, D. C. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene, in press, 2001.
56. Krehan, A., Ansuini, H., Bocher, O., Grein, S., Wirkner, U., and Pyerin, W. Transcription factors ets1, NF-κB, and Sp1 are major determinants of the promoter activity of the human protein kinase *CK2α* gene. J. Biol. Chem., *275:* 18327–18336, 2000.
57. Liang, Y. C., Huang, Y. T., Tsai, S. H., Lin-Shiau, S. Y., Chen, C. F., and Lin, J. K. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis (Lond.), *20:* 1945–1952, 1999.
58. Kumar, A., Dhawan, S., and Aggarwal, B. B. Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) inhibits TNF-induced NF-κB activation, IκB degradation, and expression of cell surface adhesion proteins in human vascular endothelial cells. Oncogene, *17:* 913–918, 1998.
59. Schoonbroodt, S., Ferriera, V., Best-Belpomme, M., Boelaert, J. R., Legrand-Poels, S., Korneer, M., and Piette, J. Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of IκB α in NF-κB activation by an oxidative stress. J. Immunol., *164:* 4292–4300, 2000.
60. Higgins, K. A., Perez, J. R., Coleman, T. A., Dorshkind, K., McComas, W. A., Sarmiento, U. M., Rosen, C. A., and Narayanan, R. Antisense inhibition of the p65 subunit of NF-κB blocks tumorigenicity and causes tumor regression. Proc. Natl. Acad. Sci. USA, *90:* 9901–9905, 1993.
61. Wu, M., Lee, H., Bellas, R. E., Schauer, S. L., Arsura, M., Katz, D., FitzGerald, M. J., Rothstein, T. L., Sherr, D. H., and Sonenshein, G. E. Inhibition of NF-κB/Rel induces apoptosis of murine B cells. EMBO J., *15:* 4682–4690, 1996.
62. Guttridge, D. C., Albanese, C., Reuther, J. Y., Pestell, R. G., and Baldwin, A. S., Jr. NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol. Cell. Biol., *19:* 5758–5799, 1999.
63. Yokoo, T., and Kitamura, M. Dual regulation of IL-1β-mediated matrix metalloproteinase-9 expression in mesangial cells by NF-κB and AP-1. Am. J. Physiol., *270:* 123–130, 1996.
64. Wang, W., Abbruzzese, J. L., Evans, D. B., and Chiao, P. J. Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated RelA. Oncogene, *18:* 4554–4563, 1999.